METHOD DEVOLOPMENT AND VALIDATION FOR ESTIMATION OF CURCUMIN IN POLY HERBAL FORMULATION BY RP-HPLC

Dissertation

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Certificate

This is to certify that the dissertation entitled "**METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF CURCUMIN IN POLY HERBAL FORMULATION BY RP-HPLC"** submitted by **Mr. RAVIKISHORE. KV (Reg.No: 26101727)** in partial fulfillment for the degree of **Master of Pharmac**y **in Pharmaceutical Analysis** under **The Tamil Nadu Dr. M.G.R. Medical University,** Chennai at **K. M College of pharmacy,** Madurai-107. It is a bonafide work carried out by him under my guidance and supervision during the academic year **2011-2012**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university or other universities.

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Ravikishore.KV…..

1. INTRODUCTION

1.1 Introduction to analytical chemistry [1]

The pharmaceutical analysis defined as "the branch of practical chemistry which deals with the resolution, separation, identification, determination and purification of a given sample of a medicine, the detection and estimation of impurities, which may be present in drug substance (or) given sample of medicine".

The substance may be a single compound or a mixture of compounds and may be in the form a tablet, pill, capsule, ampoule, liquid, mixture or an ointment.

The quality control tests involve methods which embrace chemicals, physio - chemical/ instrumental, microbiological (or) biological procedures.

The pharmaceutical analysis deals with the subject of determining the composition of material in terms of the elements or compound (drug) present in the system.

Any type of analysis involves two steps

Identification (qualitative)

Estimation (quantitative)

In qualitative analysis, a reaction is performed in such a way as to indicate the formation of a precipitate, a change of a colour, the dissolution of a precipitate/ complex formation and the evaluation of a gas.

Quantitative analysis is performed ordinarily through five steps. They are sampling, dissolution, precipitation, measurement and calculation.

Analytical Techniques [2, 3,4]

1) Titrimetric methods

a) Acid – Base titrations

b) Redox titrations

c) Precipitation titrations

d) Non-aqeous titrations

e) Diazotisation titrations

2) Gravimetric methods

a) Weighing of drug after extraction.

b) Weighing of derivative after separation.

c) Weighing residue after ignition.

3) Spectrophotometric Methods

a) Colourimetric method

b) Ultra violet method

c) Fluorimetric method

d) Flame photometry

e) Atomic absorption spectroscopy.

f) Infrared spectrophotometry

g) Raman spectroscopy

h) X-ray spectroscopy

i) Mass spectroscopy

4) Electro analytical methods

a) Potentiometry

b) Voltametry

c) Amperometry

d) Electrogravimetry

e) Conductometry

f) Polarography

5) Chromatographic methods

a) Thin layer chromatography

b) Paper chromatography

c) Column chromatography

d) Gas chromatography

e) High Performance Liquid Chromatography

6) Hyphenated techniques

a) GC-MS (Gas chromatography – Mass spectrometry)

b) LC-MS (Liquid chromatography – Mass spectrometry)

c) GC-IR (Gas chromatography – Infrared spectroscopy)

d) ICP-MS (Inductively coupled plasma – Mass spectrometry)

7) Miscellaneous methods

a) Thermal analysis

b) Kinetic techniques

c) Enzyme assay

d) Microbiological procedure

e) Biological procedure

Different instrumental methods with basic principle

Sr. N0.	MEHTOD	BASIC PRINCIPLE
A	ELECTROANALYTICAL METHODS	
1	Potentiometry	Concerned with change in electrical properties of the system measures the change in electrode potential during a chemical reaction of the system
2	Conductometry	Measures the change in electrical conductivity during a chemical reaction
3	Polarography	Measures the current at various applied potential indicating the polarization at indicator electrode
4	Amperometry	Measures the change (or decrease) in current at a fixed potential during addition of titrant
B	SPECTROSCOPIC METHODS	
1	Absorption spectroscopy (Ultraviolet – visible and infrared)	Measures the absorbance or percent transmittance during the interaction of monochromatic radiation (or particular wavelength) by the same
2	Fluorimetry	Measures the intensity of fluorescence caused by emission of electromagnetic radiation due to absorption of UV radiation
3	Flame Photometry	Measures the intensity of emitted light of particular wave length emitted by particular element
4	Turbidimetry	Measures the turbidity of a system by passing light beam in a turbid media

Sr. N0.	MEHTOD	BASIC PRINCIPLE
5	Nephlometry	Measures the opalescence of the medium by reflection of light by a colloidal solution
6	Atomic Absorption Spectroscopy	Measures the intensity of absorption when atoms absorbs the monochromatic radiation
7	X-Ray Spectroscopy	Measures the position and intensity of spectral lines during emission of X-ray spectrum by atoms under influence of X-rays
8	Refractometry	Measures the refractive index by causing refraction of light by matter
9	Polarimetry	Measures optical reaction by causing the rotation of plane polarized light
C	Mass Spectroscopy	Observe the position and intensity of signals in mass spectrum by causing the ionization of molecules
D	NMR Spectroscopy	Observe the position and intensity lines in NMR spectrum when proton interact with electromagnetic radiation in radio frequency region
E	Thermal Methods	Measures the physical parameters of the system as a function of temperature. It includes thermo gravimetry, derivative gravimetry and differential thermal analysis
F	Radiometric Methods	Measure the radioactivity either present naturally or induced artificially

PHASES IN ANALYTICAL CHEMISTRY [5]

Phase 1: Fast screening phase eg: Immuno assay, gas chromatography and Liquid chromatography.

Phase 2: Identification phase eg : GC – MS.

Phase 3: Qualification phase eg : Spectrophotometer and gas chromatography.

IMPORTANT CONSIDERATIONS IN ANALYTICAL METHODS [6]

The instrument is the most visible and exciting element of the analytical method and it is only one component of the total analysis.

- The analyst should determine the nature of the sample, the end use of the analytical results, the species to be analyzed.
- Quantitative information may include elemental composition, oxidation state, functional groups, major components, minor components, complete identification in the given sample.
- Quantitative data include accuracy, precision and range of expected analyte.
- Methods such as controlling the atmosphere to which the sample is exposed, controlling the temperature of the sample, buffering the pH of sample solutions.

SENSITIVITY AND DETECTION LIMITS [7]

TECHNIQUES	DETECTION LIMITS	IDENTIFICATION LIMITS
Gas chromatography	10^{-6}-10^{12}	-
Infrared spectrophotometry	10^{-7}	10^{-6}
Ultraviolet spectroscopy	10^{-7}	10^{-6}
N.M.R(time averaged)	10^{-7}	10^{-5}
Mass spectrometry (batch inlet)	10^{-6}	10^{-5}
Mass spectrometry (direct probe)	10^{-12}	10^{-11}

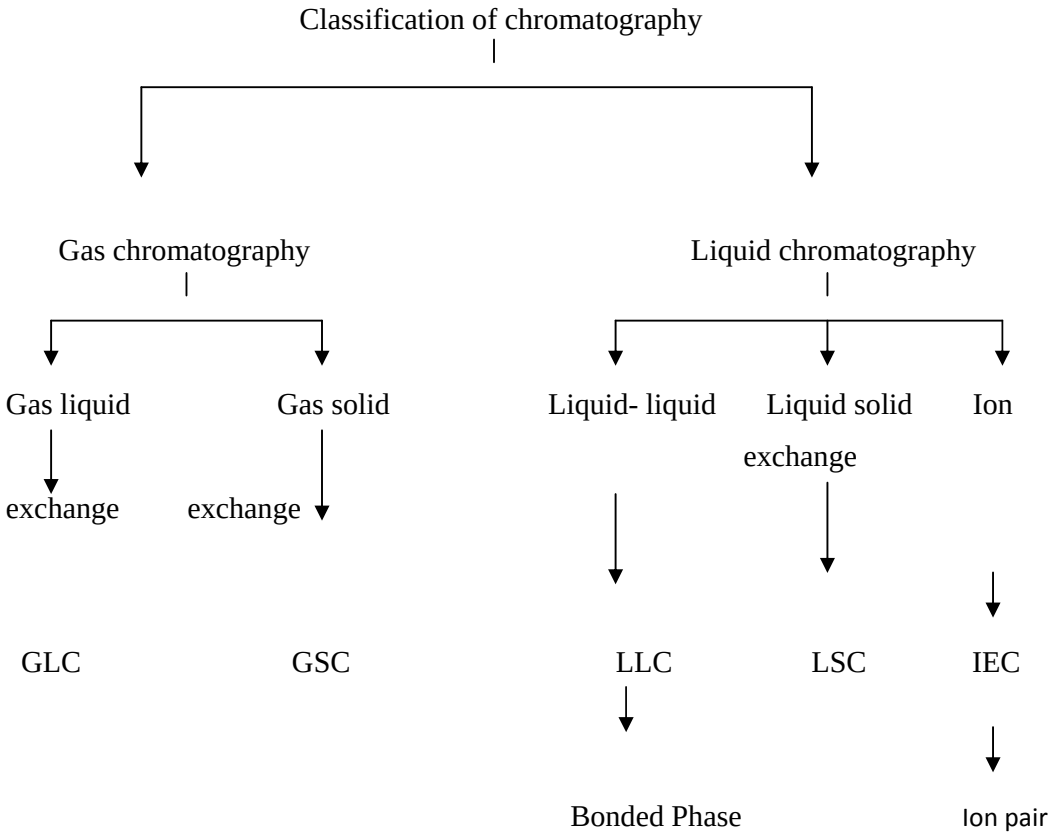
Factors Affecting the Choice of Analytical Methods [8]

- The type of analysis required.
- Problem arising from the nature of the material.
- Possible interference from components of the material other than those of interest.
- The concentration range which needs to be investigated.
- The accuracy required.
- The facilities available.
- The time required for complete analysis.
- Similar type of analysis performed.

1.2 CHROMATOGRAPHY INTRODUCTION

Chromatography is a technique for the separation of the compounds of mixtures by their continuous distribution between two phases. One is stationary phase and the other is mobile phase. As a general rule, highly polar materials are best separated using partition chromatography, while very nonpolar materials are separated using adsorption chromatography. Between extremes, either process might be applicable.

In this two mutually immiscible phases are brought in to contact one phase is stationary and other is mobile. Species in the sample undergo repeated interactions (partitions) between the mobile phase & stationary phase. The components are gradually separated in to bands in mobile phase. It is an analytical chromatographic technique that is useful for separating ions / molecule that are dissolved in a solvent.



1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [9]

High performance liquid chromatography is the fasted growing analytical technique for the analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. The rapid growth of HPLC has been facilitated by the development of reliable, moderately priced instruments and efficient columns.

High performance liquid chromatography is a convenient separation technique used for wide types of samples, with exceptional resolving power, speed and nano molecular detection levels. It is presently used in pharmaceutical research and developments in the following ways:

- To purify synthetic or natural products.
- To characterize the metabolites.
- To assay active ingredients, impurities, degradation products and in dissolution assays.
- In pharmacodynamic and pharmacokinetic studies.

Chromatography encompasses a diverse group of methods that are utilized for the separation of closely related components of mixtures. In all chromatographic separations, the sample is transported within the mobile phases, which may be a gas (GC), a liquid (LC) or a supercritical fluid chromatography (SFC). In column chromatography, the stationary phase is contained within a narrow tube through which the mobile phase is forced by gravity or under pressure. The components of the mixture to be analyzed distribute themselves between the mobile phase and stationary phase in varying proportions. Compounds that interact strongly with the stationary phase migrate very slowly with the mobile phase in contrast; compounds that are weakly retained by the packing material migrate rapidly with the mobile phase. As a consequence of the differences in mobility between the individual components of a mixture, the sample components are separated into discrete bands (or zones) that emerge from the column at specific ‘retention times. These bands may be identified qualitatively and /or further analyzed quantitatively using an appropriate detector.

Early, liquid chromatography was carried out in glass columns with diameter of 1 to 0.5 cm and length of 50 to 500 cm. The average diameter of the solid stationary phase particles was usually in the 100 to 200 micron range. Recent technology has allowed for the development of packing material with relatively small particles size diameter (3-10 μ). This technology resulted in the development of columns with very high efficiencies and consequently has involved the use of more sophisticated instrumentation to perform at increased pressures and flows hence the term High Performance Liquid Chromatography (HPLC) was given to this technique.

The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high-pressure pump.

The method is popular because it is non-destructive and may be applied to thermally liable compounds (unlike GC); it is also a very sensitive technique since it incorporates a wide choice of detection methods. With the use of post-column derivatisation methods, selectivity and detection limits are improved. HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it valuable separation tool in many scientific fields.

Advantages:

- Separation is fast and efficient
- Continuous monitoring of the column effluent
- Can be applied to the separation and analysis of very complex mixture
- Accurate quantitative measurements
- Repetitive and reproducible analysis using the same column
- Adsorption, partition, ion exchange and exclusion column separations are excellently made
- Automation of the analytical procedure and data handling
- Both aqueous and non aqueous samples can be analysed
- Providing a high degree of selectivity for specific analysis

- Determination of multiple components in a single analysis

Principle of separation in HPLC [6]

The principle of separation in normal phase and reverse phase mode is the adsorption. When a mixture of components is introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The components which have less affinity towards the stationary phase travel faster. Since no two components have the same affinity towards the stationary phase and so the components are separated.

Types of HPLC techniques

a) Based on modes of chromatography
   - Normal phase mode
   - Reverse phase mode

b) Based on principle of separation
   - Adsorption chromatography
   - Ion exchange chromatography
   - Ion pair chromatography
   - Size exclusion or gel permeation chromatography
   - Affinity chromatography
   - Chiral phase chromatography

c) Based on elution technique
   - Isocratic separation
   - Gradient separation

d) Based on scale of operation
   - Analytical HPLC
   - Preparative HPLC

Normal phase chromatography

In normal phase mode, the stationary phase (silica gel) is polar in nature and the mobile phase is non-polar. In this technique, non-polar compounds travel faster and eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because of more affinity towards stationary phase and take more time to be eluted from column.

Reverse phase chromatography

In reverse phase technique, a non polar stationary phase is used. The mobile phase is polar is in nature hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, which is advantageous. Different columns used are ODS (octadecyl silane) or C_{18}, C_8 and C_4 etc.

Adsorption chromatography

The principle of separation is adsorption. The separation of components takes place because of the difference in affinity of compounds towards stationary phase.

Ion exchange chromatography

The principle of separation is ion exchange which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions.

Ion pair chromatography

In ion pair chromatography a reverse phase column is converted temporarily in to ion exchange column by using ion pairing agents like pentane, hexane, heptanes, octane, sulphonic acid sodium salt, tetramethyl or tetraethyl ammonium hydroxide.

Size exclusion or gel permeation chromatography

In this type of chromatography, a mixture of components with different molecular sizes is separated by using gels. The gel used acts as molecular sieve and hence a mixture of substance with different molecular sizes is separated. Soft gels like dextran, agarose or polyacrylamide are used. Semi rigid gels like polystyrene, alkyl dextran in non aqueous medium are also used.

Affinity chromatography

Affinity chromatography uses the affinity of the sample with specific stationary phase. This technique is mostly used in the field of biotechnology, microbiology and biochemistry.

Chiral phase chromatography

Separation of optical isomer can be done by using chiral stationary phases. Different principles operate for different types of stationary phases and for different samples. The stationary phases used for this type of chromatography are mostly chemically bonded silica gel.

Isocratic separation

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

Gradient separation

In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Analytical HPLC

Where only analysis of the samples are done. Recovery of the samples for reusing is normally not done, since the sample used is very low. eg. µg quantities.

Preparative HPLC

Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. eg. Separation of few grams of mixtures by HPLC.

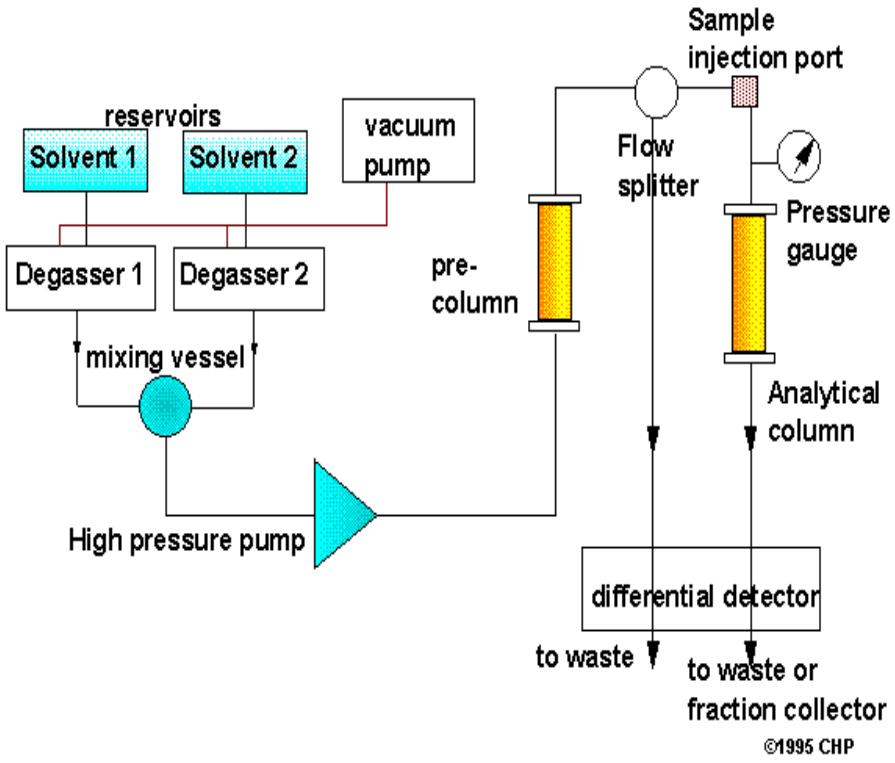
INSTRUMENTATION [6]

The general instrumentation for HPLC incorporates the following components

- There is a solvent reservoir for the mobile phase.
- The mobile phase must be delivered to the column by some type of pump. To obtain separations either based on short analysis time or under optimum pressure, a wide range of pressure and flows is desirable. The pumping system must be pulse-free or else have a pulse damper to avoid generating base line instability in the detector.
- Sampling valves or loops are used to inject the sample into the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of the mobile phase (if possible) to eliminate an unnecessary solvent peak .
- A head of the separation column there may be a guard column or an in –line filter to prevent contamination of the main column by small particles.
- To measure column inlet pressure a pressure gauge is inserted in front of the separation column.
- The separation column contains the packing needed to accomplish the desired HPLC separation. These may be silicas for adsorption chromatography, bonded phases for liquid-liquid chromatography, ion-exchange chromatography, gels of specific porosity for exclusion chromatography or some other unique packing for a particular separation method.
- A detector with some type of data handling devise completes the basic instrumentation.

The essential parts of the high performance liquid chromatography are the solvent reservoir, tubing, pump, Injection device, column, detector and recorders.

HPLC instrumentations



Parts of HPLC [10]

A) Solvent reservoir

B) Tubing

C) Pumps

D) Sample injection system

E) Column and fittings.

F) Detectors

G) Recorders

A) Solvent reservoir

The solvent reservoir should posses several characteristics. The composition of the reservoir should render it inert to a variety of aqueous and non aqueous mobile phases and should be clean. The volume capacity of reservoir of the reservoir should be greater than 500 ml. Since mobile phase velocities are typically 1-2 ml/min this volume provides for 4 hr of operation. It should have cap that allow the tubing inlet line to pass through it in to reservoir. In many cases, aqueous solvents and some organic solvents are degassed prior to use. This is done to prevent formation of gas bubbles in the detectors. Stirring of mobile phase under the vacuum or sparing with helium gas is effective ways of degassing. If the reservoir made up of the glass, precaution should be taken to prevent to solvent spills in case of breakage of reservoir. The reservoir should be placed in plastic containers.

B) Tubing

The nature of the tubing is used to connect all the parts of system deserves some attention. The inside diameter of the tubing prior to the injection device is not critical but tubing should be inert, have the ability to withstand pressure and be able to carry sufficient volume. The length of the tubing, L that can be used with a maximum band width increase of 5% can be calculated from.

$$L = \frac{40\, V_R^2\, D_m}{\Pi\, F\, d^4\, N}$$

Where,

V_R – Is the volume retention in ml.

D_m – Is the solute diffusivity in cm^2/sec

F – Is the flow rate in ml/sec.

d – Is the diameter of tube in cm

N – Is the column plate number.

C) Pumps

There are a number of different types of pumps that can provide the necessary pressure and flow- rate required by the modern liquid chromatography. In the early years of the LC renaissance, there were two types of pump in common use; they were the pneumatic amplification and the syringe pump, which was simply a large, strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern chromatographs are fitted with reciprocating pump with either pistons or diaphragms.

Pneumatic pump

The pneumatic pump has a much larger flow capacity than the piston type pumps but, nowadays, is largely used for column packing and not for general analysis. The pneumatic pump can provide extremely high pressures and is relatively inexpensive, but the high pressure models are a little cumbersome and, at high flow rates, can consume considerable quantities of compressed air.

Syringe pump

The syringe pump is a large, electrically operated simulation of a hypodermic syringe. Although used in the early days of LC renaissance, it is rarely used today as, due to its design, it can provide only a limited pressure and the volume of mobile phase available pump is able for use is restricted to the pump volume. Unless the separation is stopped while the pump is refilled and the development subsequently continued. It only elute solutes that have retention volumes equal or less than the pump capacity.

D) Sample injecting system [(8)]

Introduction of the sample is generally achieved is one of two ways, either by using syringe injection or through a sampling valve. Septum injectors allow sample introduction by a high pressure syringe through a self sealing elastomer septum. One of the problems associated with septum injectors is the leaching effect of the mobile phase in contact with the septum, which may give rise to ghost peaks. In general, syringe injection for HPLC is more troublesome than in gas chromatography.

Although the problems associated with septum injectors can be eliminated by using stop flow septum less injection. Currently the most widely used devices in commercial chromatographs are the micro volume sampling valves. Which enable sample to be introduced reproducibly in to pressurized columns without significantly interrupting the flow of the mobile phase. The sample is loaded at atmospheric pressure in to an external loop in the valve and introduced into the mobile phase by an appropriate rotation of the valve. The volume of sample introduced, ranging from 2 µl to over 100 µl, may be varied by changing the volume of the sample loop or by using special variable volumes sample valves. Automatic sample injectors are also available which allow unattended operation of the instrument. Valve injection is preferred for quantitative work because of its higher precision compared to syringe injection.

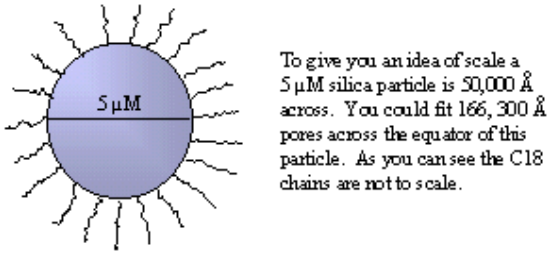
E) Column

Since columns are tubular, column dimensions usually take the following format, internal diameter X length (4.6mm X 250mm). As a mass spectroscopist you will encounter columns ranging in internal diameter from 0.050 to 4.6 mm or even larger if you are performing large scale preparative chromatography. For mass spectrometry a short reverse phase column will work nearly as well as a longer column and this is an important fact because shorter columns are generally cheaper and generate less back pressure. Why is less back pressure important? If a column runs at low pressure it allows the user more flexibility to adjust the flow rate. Sometimes shorter columns are used to do fast chromatography at higher than normal flow rates. In terms of length we routinely run 100 mm columns; however 50 mm or 30 mm columns may be adequate for many LC/MS separation needs.

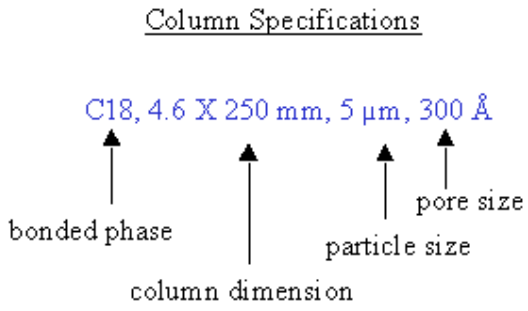
The most common columns are packed with silica particles. The beads or particles are generally characterized by particle and pore size. Particle sizes generally range between 3 and 50 microns, with 5 µm particles being the most popular for peptides. Larger particles will generate less system pressure and smaller particles will generate more pressure. The smaller particles generally give higher separation efficiencies. The particle pore size is measured in angstroms and generally

range between 100-1000 angstroms. 300 angstroms is the most popular pore size for proteins and peptides and 100 angstroms is the most common for small molecules.

Silica is the most common particle material. Since silica dissolves at high pH, it is not recommended to use solvents that exceed pH 7. However, recently some manufactures have introduced silica based technology that is more resistant to high pH, it is important to take note of the manufactures suggested use recommendations.



The stationary phase is generally made up of hydrophobic alkyl chains (-CH_2-CH_2-CH_2-CH_3) that interact with the analyte. There are three common chain lengths, C_4, C_8 and C_{18}. C_4 is generally used for proteins and C_{18} is generally used to capture peptides or small molecules. The idea here is that the larger protein molecule will likely have more hydrophobic moieties to interact with the column and thus a shorter chain length is more appropriate. Peptides are smaller and need the more hydrophobic longer chain lengths to be captured, so C_8 and C_{18} are used for peptides or small molecules. Here is an interesting note: Observations have been made that C_8 columns are actually better for capturing smaller hydrophilic peptides, the theory here is that the longer C_{18} chains lay down during the early aqueous period of the gradient and the more hydrophilic peptides are not captured. We use C_8 routinely for all peptide work and this particular alkyl chain length works equally well if not better than C_{18} for all peptides.



F) Detectors

Detector is the eye of LC system and measures the compounds after the separation on the column. Before the first sample is injected, during method development the chromatographer must ensure that the detector so selected is capable of responding to changes in the concentration of all the components in the sample with adequate sensitivity even to measure trace substances.

There are basically two types of detectors.

-Bulk property detectors

-Solute property detectors

The Bulk property detectors functions based on some bulk property of the eluent, such as refractive index and are not suitable for gradient elution and are usually less sensitive than solute property detectors. The solute property detectors perform by measuring some type of physical or chemical property. Solute property detectors are significantly more sensitive than bulk property detectors. Over 70% of the HPLC detectors are solute property detectors.

Various detectors are used in HPLC they are,

(1) UV absorbance detector
(2) Fluorescence detector
(3) Electrochemical detector
(4) Conductivity detectors
(5) Refractive index detector
(6) Mass spectrometer.

UV Absorbance detectors:

The mobile phase is passed through a small flow cell, where the radiation beam of a UV/visible photometer or spectrophotometer is located. As a UV-absorbing solute passes through the flow cell, a signal is generated that is proportional to the solute concentration. Only UV-absorbing compounds, such as alkenes, aromatics and compounds that have multiple bonds between C and O, N or S are detected. The

mobile phase components should be selected carefully so that they absorb little or no radiation.

Absorption of radiation as a function of concentration, c is described by the beer-lamberts law.

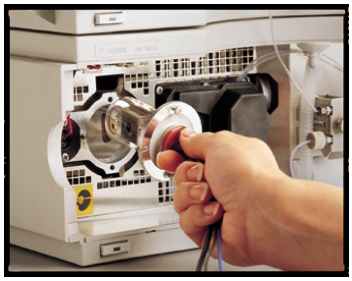
$$A = \varepsilon bc$$

Where, A = absorbance,

ε = molar extinction coefficient,

b = flow cell path length.

Three types of absorbance detectors are available: fixed-wavelength, variable wavelength, and photodiode array. A fixed – wavelength detector uses a light source that emits maximum light intensity at one or several discrete wavelength that is isolate by appropriate filters. For example, with a commonly used mercury lamp, the wavelengths of 254, 280, 313, 334 and 365 nm can be selected. A fixed-wavelength detector is the most sensitive and least expensive of the three, but it is inflexible in wavelength selection. A variable-wavelength detector uses a relatively wide band-pass UV/visible spectrophotometer. It offers an increased number of UV and visible wavelength, but it is more expensive than the fixed-wavelength detector. In order to generate real-time spectra for each solute as it is eluted, a photodiode array is used.



UV LAMP

UV DETECTOR

G) **Computer, Integrator or Recorder:**

A data collection device such as a computer, integrator or recorder is connected to the detector. It takes the electronic signal produced by the detector and plots it as a chromatogram, which can be evaluated by the user. Recorders are rarely used today because they are unable to integrate the data. Both integrators and

computers can integrate the peaks in the chromatograms and computers have the further advantage that they electronically save chromatograms for later evaluation

Isocratic and gradient elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed **isocratic** (meaning *constant composition*). The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a **gradient elution**. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; **A** is the "weak" solvent which allows the solute to elute only slowly, while **B** is the" strong" solvent which rapidly elutes the solutes from the column. Solvent **A** is often water, while **B** is an organic solvent miscible with water, such as acetonitrile, methanol, THF or isopropanol.

Normal phase chromatography:

Normal phase HPLC (NP-HPLC) or adsorption chromatography, this method separates analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte

molecule, but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. This behavior is somewhat peculiar to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

Reverse phase chromatography:

Reversed-phase chromatography (RPC or RP-HPLC) includes any chromatographic method that uses a non-polar stationary phase. Liquid chromatography was done on non-modified silica or alumina with a hydrophilic surface chemistry and a stronger affinity for polar compounds - hence it was considered "normal". The introduction of alkyl chains bonded covalently to the support surface reversed the elution order. Now polar compounds are eluted first while non-polar compounds are retained – hence called as "reversed phase" chromatography. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically changing the polarity of the mobile phase during the course of the analysis.

Retention characteristic in reverse phase chromatography:

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -

OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligand's alkyl chains and can have problems entering the pores of the stationary phase. Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C≡C as the double or triple bond is shorter than a single C-C-bond.

Stationary phases in reverse phase chromatography:

Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. The most popular column is a C_{18} bonded silica (USP classification L1) followed by C_8 bonded silica (L7), pure silica (L3), cyano bonded silica (L10) and phenyl bonded silica (L11). That C_{18}, C_8 and phenyl are dedicated reversed phase packings while cyano columns can be used in a reversed phase mode depending on analyte and mobile phase conditions. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping)

Mobile phase consideration in reverse phase chromatography:

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column. The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF).

Other Solvents can be used such as ethanol, 2-propanol (iso-propyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

VARIOUS METHODS OF QUANTITATIVE ANALYSIS IN HPLC [11, 12]

The sample or solute is analysed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width are constant and are strongly affected by the sample injection techniques. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute.

a) Calibration by Standards

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear.

$$X = K \times Area.$$

Where, X = Concentration of solute.

K = Proportionality constant (slope of the curve).

In this evaluation method only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecular type of compounds.

b) Internal Standard Method

In this technique a known quantity of the internal standard is chromatographed and area versus concentration is ascertained. Then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operations.

The peak area of the standard in the sample run is compared with the peak area when the standard is run separately. This ratio serves as a correction factor for variation in sample size, for losses in any preliminary pretreatment operations or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components, must not interfere with the sample components and must never be present in samples.

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area ot internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{Concentration of standard}$$

c) Area Normalization

The technique is often used for the sample having identical components. It is used to evaluate the absolute purity of the sample. The procedure is to total up the areas under all peaks and then calculate the percentage of the total area that is contributed by the compound of interest. For this method the entire sample must be eluted, all components must be separated and each peak must be completely resolved.

d) Standard Addition Method

If only few samples are to be chromatographed, it is possible to employ the method of standard addition(s).The chromatogram of the unknown analyte is recorded, then a known amount of analyte (s) is added and the chromatogram is repeated using same reagents, instruments and same conditions. From the increase in the peak area (or) peak height, the original concentration can be computed by interpolation.

The detector response must be a linear function of analyte concentration and yield no signal at zero concentration of the analyte. Sufficient time must elapse between addition of the standard and actual analysis to allow equilibrium of added standard with any matrix interferon.

If an instrumental reading (area/height) 'R_x' is obtained, from a sample of unknown 'x' and a reading 'R_t' is obtained from the sample to which a known concentration 'a' of analyte has been added, then 'x' can be calculated from.

$$\frac{x}{x+a} = \frac{R_x}{R_t}$$

A correction for dilution must be made if the amount of standard added changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

e) External Standard Method

It employs a separate injection of a fixed volume of sample and standard solution. The peaks are integrated and concentration is calculated.

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

The selection of suitable chromatographic (HPLC) system for a given mixtures of solutes cannot be made with certainty and must be confirmed by experiment. If the chemical nature of the sample components is known, then the phase system can be selected from the literature references. If nothing is known about the chemical nature of sample, then the sample solubility will give some indication as to which chromatographic method to employ. The essential parts of high performance liquid chromatographic system are solvent reservoir, pump, injection port, column, detector and recorders.

1.4 SYSTEM SUITABILITY PARAMETERS [12]

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

The parameters that are affected by the changes in chromatographic conditions are,

- Column capacity factor (K_A)
- Resolution (R_s)
- Selectivity (α)
- Column efficiency (N)
- Peak asymmetry factor (A_s)

i) Column capacity factor (K_A)

The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter.

The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$K_A = \frac{V_A - V_0}{V_0} = \frac{t_A - t_0}{t_0}$$

Where,

V_A = Elution volume of A

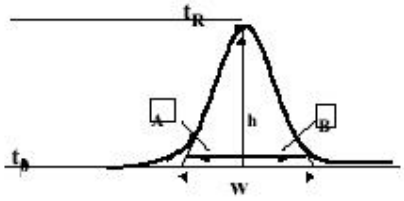
V_0 = Elution volume of a non retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes. Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times $\left(\frac{t_A - t_o}{t_B - t_o}\right)$ is better when data need to be transferred between different chromatographs.

The values of k' of individual bands increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$



ii) Resolution (R_s)

The resolution, R_s of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 2.0. It is calculated by using the formula,

$$R_f = \frac{Rt_2 - Rt_1}{0.5\,(W_1 + W_2)}$$

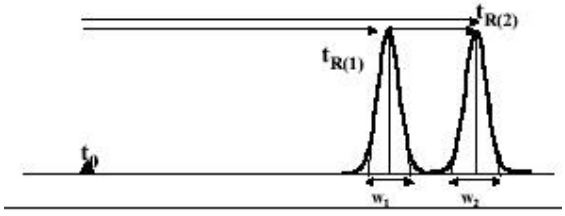
Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2

W_1 and W_2 are peak widths of components 1 and 2.

EXPERIMENTAL RESOLUTION

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2\,(W_1 + W_2)}$$

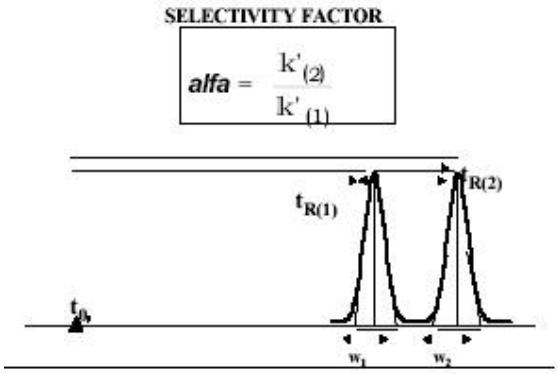


iv) Selectivity (α)

The selectivity (or separation factor),α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



v) Column efficiency

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\frac{Rt^2}{W^2}$$

Where, Rt is the retention time and W is the peak width.

vi) Peak asymmetry factor (A_s)

Peak asymmetry factor, A_s can be used as a criterion of column performance. The peak half width b of a peak at 10 % of the peak height, divided by the corresponding front half width a gives the asymmetry factor.

STATISTICAL PARAMETERS [13]

Regression equation:

The linear relationship is characterized by attendency of the points of the scattered diagram to cluster along a straight line, known as the regression line.

$$Y=a + bX$$

It is used to describe the dependence of one characteristic (Y) up on the other characterstic (X), both X,Y represent values of two characters, a,b are two constants it will be evident that two regression lines can be computed for every set of data-one each to describe the dependence of one character to another. b is known as regressive coefficients which shows change expected in Y for unit change in X, it is dependence of Y & X; b is the regressive coefficient of Y& X.

The regressive coefficient of b is estimated,

$$b = \frac{\Sigma\left(x - \bar{x}\right)\left(y - \bar{y}\right)}{\Sigma\left(x - \bar{x}\right)^2}$$

b = the slope of the regression line and is calculated by this formula

x = an arbitrarily chosen value of the predictor variable for which the corresponding value of the criterion variable is desired.

Correlation coefficient:

A measure of the strength of the relationship between two variables is provided by the coefficient of correlation, denoted by r, if the relationship between the two variables is of the linear form. It is also called the coefficient of linear correlation

Pearson's correlation:

The correlation coefficient calculation for data values should be +1 or -1

Where the values of Correlation coefficient are +1 – positive

Correlation coefficient is -1 – negative

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}}$$

Where,

X – Value of one character

Y – Value of another character

Standard deviation:

It is the square root of the average of the squared deviations of the observations. From the arithmetic mean, it is used for measures of dispersion.

It is denoted by

Standard Deviation = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$

R.S.D (%) = $\frac{S.D}{\bar{x}} \times 100$

Where Σ = Sum of observations

$\bar{x}$ = Mean or arithmetic average ($\Sigma x / n$)

x = Individual observed value

$x - \bar{x}$ = Deviation of a value from the mean

n = Number of observations

Standard error of mean (S.E):

The population of standard deviation is not given, but the size of s is large, so the sample standard deviation is representing the population of standard deviation.

$$S.E. = \frac{S.D}{\sqrt{n}}$$

Where,

S.D = Standard deviation

n = no. of observation

1.5 ICH GUIDELINES FOR ANALYTICAL METHOD VALIDATION[14]

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated,

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instruments with different characteristics.
- Whenever the method is changed and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures.

REASONS FOR VALIDATION

1. Enables scientists to communicate scientifically and effectively on technical matters.
2. Setting standards of evaluation procedures for checking complaints and taking remedial measures
3. Retrospective validation is useful for trend comparison of results compliance to cGMP/GLP.
4. Closer interaction with pharmacopoeia harmonization particularly in respect of impurities determination and their limits.
5. For taking appropriate action in case of non – compliance.
6. To provide high degree of confidence that the same level of quality is consistently built into each unit of finished product from batch to batch.

   As quality control process is not static, some form of validation / Verification should continue till the validated process is in use.

The parameters as defined by the ICH and by other organizations

- Specificity
- Selectivity
- Precision
  - Repeatability
  - Intermediate precision
  - reproducibility
- Accuracy
- Linearity
- Range
- Limit of detection
- Limit of quantification
- Robustness
- Ruggedness

A) Specificity and selectivity

i. Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

ii. Specificity for an assay ensures that the signal measured comes from the substance of interest and that there is no interference from excipient and/or degradation products and/or impurities.

iii. Determination of this can be carried out by assessing the peak identity and purity.

iv. Diode array detectors can facilitate the development and validation of HPLC assays. Spectra] data obtained from diode array detectors, effectively supplement the retention time data for peak identification, also spectral manipulation often provides information about the peak purity. The table below lists several of the techniques available for assessing peak identity and purity.

v. The purity index is a measure of the peak's relative purity, measured using a full comparison of spectral data for the leading and training edge of the peak. A value of 1.5 is commonly accepted to indicate a pure peak but >1.5 would indicate the presence of an impurity.

B) Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed at the variance, standard deviation or coefficient of variation of a series of measurements.

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Precision is a measure of the reproducibility of the *whole* analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (ie between 6 - 1 0). The precision is then expressed as the relative standard deviation

$$\%RSD = \frac{\text{STD dev x } 100\%}{\text{Mean}}$$

Repeatability

Express the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra - assay precision. It should be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g. three concentration/three replicates each) or a minimum of determinations at 100% of the test concentration.

Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance inclusion of procedures in Pharmacopoeias.

C) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or on an accepted reference value and the value found.

Assay

- Assay of Active Substance
- Assay of Medicinal products

Several methods are available to determine the accuracy.

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material).
b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure)
c) Application of the analytical procedure to synthetic mixtures of the product components to which known quantities of the substance to be analyzed have been added.

Impurity (Quantification)

Accuracy should be assessed on sample (substance /products) spiked with known amounts of impurities. It should be clear how the individual or total impurities are to be determined.

E.g. Weight / Weight or area percent.

D) Linearity and range

Linearity of an analytical procedure is its ability (with in a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods. For example, calculation of a regression line by the method of least square. Therefore data from regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

Range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample including these concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The following minimum specified ranges should be considered

- For the assay of an active substance or a finished product normally from 80-120 percent of the test concentration.
- For the content uniformity, covering a minimum of 70-130 percent of the test concentration.
- For dissolution testing, 20% over the specified range (e.g.), If the specifications for a controlled release product cover a region from 20% after 1 hour, upto 90% after 24 hours, the validated range would be 0-110% of label claim.
- For the determination of an impurity, the reporting level of an impurity to 120% of the specifications.

E) Limit of Detection

The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

i. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may be used with instrumental methods.

ii. Based on signal to noise ratio

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio 3 or 2:1is generally considered acceptable for estimating the detection limit.

iii. Based on the standard deviation of the response and the slope

The detection limit (DL) may be expressed as

$$D\ L = 3.3\sigma/S$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope, S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

a. Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

b. Based on the calibration curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercept of regression lines may be used as the standard deviation

F) Limit of Quantification

The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision

i. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

ii. Based on Signal- to-Noise ratio

Determination of the Signal-to-Noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical Signal- to-Noise ratio is 10:1.

iii. Based on the Standard Deviation of the Response and the slope

The quantification limit (Q L) may be expressed as

$$QL = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimation may be carried out in a variety of ways including,

a. Based on standard deviation of the blank

Measurement of the magnitude of analytical background response is performed by an appropriate number of blank samples and calculating the standard deviation of these responses.

b. Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation

G) Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Examples of typical variations are

- Stability of analytical solutions.
- Extraction time.

In case of liquid chromatography, examples of typical are

- Influence of variation of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and / or suppliers),
- Temperature,
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

F) Ruggedness

The United States pharmacopoeia (USP) define ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different labs, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst.

2.1 PLANT PROFILE

Curcuma longa **(Turmeric)** is dried rhizomes of *Curcuma longa* Linn, family *Zingiberaceae*. It is native to tropical South Asia and also cultivated all states in India, needs temperatures between 20 °C and 30 °C and a considerable amount of annual rainfall to thrive.

Synonyms:

Turmeric, Indian saffron, Curcuma, Haridra.

Other names:

San	: Haridra, Nisa
Bengal, Hindi	: Haldi
Gujarat	: Halad
Kannada& Tamil	: manjal
Telugu	: Pasupu

Scientific classification:

Kingdom: Plantae

Order: Zingibaracea

Family: Zingibarecea

Genes: Curcumalong

Species: C.Long.

Curcuma Longa plant

Description:

Colour : yellowish – Brown colour,

Odour : Characteristic odour,

Taste : Bitter Taste,

Shape : Round, Cylindrical.

Chemical constituents:

Curcumalonga contain 5% of Diaryl heptanoid colouring material know as Curcuminoids. They are Curcumin, Demethoxy Curcumin, Bis- Demethoxy Curcumin. All pharmacological actions Curcumalonga due to Curcumin only.

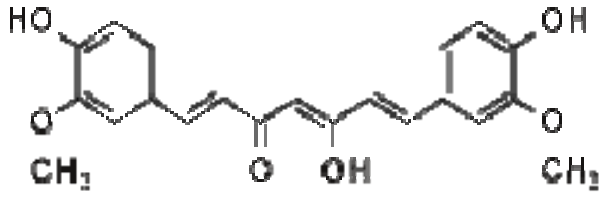
- Curcuminoids 0.8 to 2%
- Starch 30 to 40 %
- Volatile oil 3 to 12%

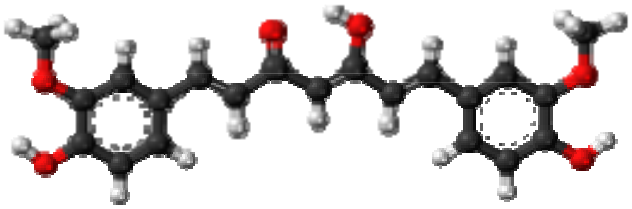
2.2 DRUG PROFILE

DRUG NAME: CURCUMIN

Curcumin is the principal curcuminoid of popular Indian spice Turmeric, which is a member of Ginger family *(Zingiberaceae*). The other two curcuminoids are Demethoxy and Bis-demethxy Curcumin. The curcuminoids are natural phenols and responsible for the yellow colour of turmeric. Curcumin can exist in several tautomeric forms. The enol form is more energetically stable in the solid phase and in solution[(15)].

DRUG STRUCTURE:





(1*E*, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-Dione.

CHEMICAL FORMULA	**:** $C_{21}H_{20}O_6$
MOLECULAR MASS	**:** 368.38 g/mol
APPEARANCE	**:** Bright yellow-orange powder
MELTING POINT	**:** 183 °C (361 °F) (456.15 K)
SOLUBILITY	**:** Insoluble in Water and soluble in THF, Acetonitrile, methanol.

PHARMACO KINETICS:

Pharmacokinetic studies in animal have demonstrated that 40-85 percent of an oral dose of Curcumin passes through the gastrointestinal track uncharged, with most of the absorbed flavonoid being metabolized in the intestinal mucosa and liver. Due to its low rate of absorption, Curcumin is often formulated with bromelain for increased absorption and enhanced anti-inflammatory effect.

MECHANISAM OF ACTION:

Anti oxidant effect

Water- and fat-soluble extracts of turmeric and its curcumin component exhibit strong antioxidant activity, comparable to vitamins C and E. A study of ischemia in the feline heart demonstrated that curcumin pretreatment decreased ischemia-induced changes in the heart. An *in vitro* study measuring the effect of curcumin on endothelial heme oxygenase-1, an inducible stress protein, was conducted utilizing bovine aortic endothelial cells. Incubation (18 hours) with curcumin resulted in enhanced cellular resistance to oxidative damage.

Hepato protective effect

Turmeric has been found to have a hepatoprotective characteristic similar to silymanrin. Animal studies have demonstrated turmeric's hepatoprotective effects from a variety of hepatotoxic insults, including carbon tetrachloride (CCl_4), galctosamine acetaminophen and Aspergillus aflotoxin. Turmeric's hepatoprotective effect is mainly a result of its antioxidant properties, as well as its ability to decrease the formation of proinflamatory cytokines. Turmeric and Curcumin also reserved billiary hyperplasia, fatty changes and necrosis induced by aflatoxin production.

Ant carcinogenic Effects

Animal studies involving rats and mice, as well as *in vitro* studies utilizing human cell lines, have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth. In two studies of colon and prostate cancer, curcumin inhibited cell proliferation and tumor growth. Turmeric and curcumin are also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both *in vitro* and *in vivo* studies. The anticarcinogenic effects of turmeric and curcumin are due to direct antioxidant

and free-radical scavenging effects as well as their ability to indirectly increase glutathione levels, thereby aiding in hepatic detoxification.

Gastrointestinal Effects

Constituents of *Curcuma longa* exert several protective effects on the gastrointestinal tract. Sodium curcuminate inhibited intestinal spasm and p-tolymethylcarbinol, a turmeric component, increased gastrin, secretin, bicarbonate and pancreatic enzyme secretion. Turmeric has also been shown to inhibit ulcer formation caused by stress, alcohol, indomethacin, pyloric ligation and reserpine, significantly increasing gastric wall mucus in rats subjected to these gastrointestinal insults.

Anti microbial effect

Turmeric extract and the essential oil of *Curcuma longa* inhibit the growth of a variety of bacteria, parasites and pathogenic fungi. A study of chicks infected with the caecal parasite *Eimera maxima* demonstrated that diets supplemented with 1-percent turmeric resulted in a reduction in small intestinal lesion scores and improved weight gain. Another animal study, in which guinea pigs were infected with dermatophytes, pathogenic molds or yeast, found that topically applied turmeric oil did not inhibit dermatophytes and pathogenic fungi, but neither curcumin nor turmeric oil affected the yeast isolates. Improvements in lesions were observed in the dermatophyte and fungi-infected guinea pigs and at seven days post-turmeric application the lesions disappeared. Curcumin has also been found to have moderate activity against *Plasmodium falciparum* and *Leishmania major* organisms.

Cardiovascular Effects

Turmeric's protective effects on the cardiovascular system include lowering cholesterol and triglyceride levels, decreasing susceptibility of low density lipoprotein (LDL) to lipid peroxidation and inhibiting platelet aggregation. These effects have been noted even with low doses of turmeric. A study of 18 atherosclerotic rabbits given low-dose (1.6-3.2 mg/kg body weight daily) turmeric extract demonstrated decreased susceptibility of LDL to lipid peroxidation, in addition to lower plasma cholesterol and triglyceride levels. The higher dose did not decrease lipid peroxidation of LDL, but cholesterol and triglyceride level decreases were noted, although to a lesser degree than with the lower dose. Turmeric extract's effect on cholesterol levels may be due to decreased cholesterol uptake in the intestines and increased conversion

of cholesterol to bile acids in the liver. Inhibition of platelet aggregation by *C. longa* constituents is thought to be via potentiation of prostacyclin synthesis and inhibition of thromboxane synthesis.

Dosage

Doses of 500-8,000 mg of powdered turmeric per day have been used in human studies. Standardized extracts are typically used in lower amounts, in the 250-2,000 mg range.

Side Effects and Toxicity

No significant toxicity has been reported following either acute or chronic administration of turmeric extracts at standard doses. At very high doses (100 mg/kg body weight), curcumin may be ulcerogenic in animals, as evidenced by one rat study.

Contra indications

Those taking blood thinner (Coumadin, etc) should avoid Curcumin, as it may add to their anti- clotting effects. Extended use is not recommended, as it may result in stomach distress. Normal therapeutic doses of Curcumin protect from ulcers, but at very high doses, it may induce ulcer. Curcumin is not recommended for persons with biliary track obstruction, as the Curcumin stimulates bile secretion.

This is why it is very important to take only the recommended dose of this herbal remedy. Since Curcumin is considered a uterine stimulant, pregnant women should not use it. Curcumin is said to work best when taken on an empty stomach.

Medicinal uses

Anti microbial, Anti inflammatory, Hepato protective, Anti carcinogenic, Anti bacterial, Anti oxidant, Anti mutagenic, lower blood cholesterol level.

3. LITERATURE REVIEW

1. AK Dwivedi, et al [16], developed a combined thin-layer chromatography-densitometry for the quantitation of curcumin in pharmaceutical dosage forms and in serum, A new TLC Densitometric method for the quantitative estimation of a nonsteroidal anti-inflammatory agent curcumin in bulk drug samples, pharmaceutical dosage forms and in human serum had been worked out. The assay combined TLC on Silicagel 60 F254 with spot visualisation by single wavelength reflection mode, excitation 265 nm and emission UV D_2 filter and densitometry.

2. Jayaprakasha_GK et al [17], reported a HPLC Method for the determination of Curcumin, Demethoxycurcumin, and Bis-demethoxycurcumin. HPLC separation was performed on a C_{18} column using three solvents, methanol, 2% AcOH, and Acetonitrile (30:50:20) with detection at 425 nm. Four different commercially available varieties of turmeric samples (Salem, Erode, Balasore, and local market samples) were analyzed to detect the percentage of these three curcuminoids. The percentages of Curcumin, Demethoxycurcumin and Bis-demethoxycurcumin as estimated using their calibration curves were found to be 1.06 +/- 0.061 to 5.65 +/- 0.040, 0.83 +/- 0.047 to 3.36 +/- 0.040, and 0.42 +/- 0.036 to 2.16 +/- 0.06, respectively, in four different samples. The total percentages of curcuminoids were 2.34 +/- 0.171 to 9.18 +/- 0.232%.

3. Jayant K. Verma et al [18], reported a Rapid HPTLC method for identification and quantification of curcumin, piperine and thymol in an ayurvedic formulation, Chromatography was performed on silica gel 60 F_{254} plates with toluene-ethyl acetate-methanol, 9 + 1 + 0.5(*v/v*), as mobile phase. Plates were developed to a distance of 8 cm at room temperature without chamber saturation. The plates were scanned and the compounds were quantified at their wavelengths of maximum absorption, 420, 333 and 277 nm for Curcumin, piperine and thymol respectively. The respective R_f values of Curcumin, piperine and thymol were 0.23, 0.30 and 0.64. Response was a linear function of the amount applied to the plate in the ranges 50–250 ng, 10–60 ng and 100–700 ng for curcumin, piperine and thymol respectively. LOD for Curcumin, piperine and thymol were 25, 5 and 50 ng respectively. The mean results from assay of Curcumin, piperine and thymol in the ayurvedic formulation were found to be 0.85, 12.93 and 3.29 mg g^{-1} respectively. The respective covariance for Curcumin, piperine and thymol was 0.78, 0.51 and 0.69% respectively. Recovery was 100.41, 99.52 and 101.21% for Curcumin, piperine and thymol respectively.

Rapid identification of curcumin, piperine and thymol was also possible by spraying the plate with anisaldehyde in sulfuric acid reagent.

4. Zengshuan Ma, et al [19], reported High-performance liquid chromatography analysis of Curcumin in rat plasma application to pharmacokinetics of polymeric micellar formulation of Curcumin. Separation was achieved on a Waters μ Bondapak™ C_{18} column (3.9 × 300 mm, 5 μm) using Acetonitrile (55%) and citric buffer, pH 3.0 (45%) as the mobile phase (flow rate = 1.0 mL/min). The UV detection wavelength was 300 and 428 nm for IS and Curcumin, respectively. The extraction efficiencies were 97.08, 95.69 and 94.90% for 50, 200 and 1000 ng/mL of Curcumin in rat plasma, respectively. The calibration curve was linear over the range 0.02–1 μg/mL with a correlation coefficient of $r^2 > 0.999$. The intra and inter-day coefficients of variation were less than 13% and mean intra- and inter-day errors were less than ±6% at 50, 200 and 1000 ng/mL of Curcumin.

5. M paramasivam , et al [20], standardized the occurrence of curcuminoids in Curcumalonga by using HPTLC. A simple performance thin layer chromatographic (HPTLC) method had been developed for the simultaneous determination of the pharmacologically import active curcuminoids such as Curcumin DMC and BDMC in Curcumalonga L. The assay combined the separation and quantification of the analytes on silica gel 60 GF245 HPTLC plates with visualization under UV and scanned at 425 nm.

6. Tanaka K et al [21], developed a near infrared spectroscopic analysis for Quantization of curcuminoids in curcuma rhizome. This study was investigated a nondestructive and rapid quantization of the curcuminoids, including Curcumin, Demethoxycurcumin, and Bis-demethoxycurcumin, present in turmeric using near-infrared (NIR) spectroscopy and multivariate statistics. In the second derivatives of the NIR spectra of turmeric samples, two characteristic absorptions of curcuminoids were detected around 1700 and 2300-2320 nm. Partial least-squares regression (PLS-R) analysis was applied to the NIR spectra obtained from 34 turmeric samples and PLS models for the quantization of Curcumin, Demethoxycurcumin, Bis-demethoxycurcumin and total curcuminoid contents in the pulverized turmeric samples were constructed. Combination usage of the Standard Normal Variate (SNV) and second derivatives were obviously superior to other preprocessing methods. The

lowest root mean squared error of cross-validation (RMSECV) values were detected at 6, 6, 6, and 6 PLS factors, for the quantitative subjects Curcumin, Demethoxycurcumin, Bis-demethoxycurcumin and total curcuminoid contents.

7. Zhang J, et al [22], developed a simple HPLC-fluorescence method for quantitation of curcuminoids and its application to turmeric products**.** This method involved a simple ultrasonic extraction with methanol as a pretreatment of turmeric products. The separation of curcuminoids and 2,5-xylenol (internal standard) was achieved within 30 min on a Cadenza CD-C_{18} column (250 x 4.6 mm; i.d., 3 micron) with a mixture of acetate buffer and CH_3 CN. The calibration curves of standard curcuminoids showed good linearities of more than 0.993 of the correlation coefficient. The instrumental detection limits for C, DMC and BDMC (signal-to-noise ratio = 3) were 1.5, 0.9 and 0.09 ng mL(1), respectively. The relative standard deviations of intra and inter-day assays by curcuminoids spiked to turmeric powder were less than 6.1%. The proposed method was successfully applied to determine curcuminoids in commercial turmeric products, such as turmeric powders, a tablet, a dressing, a beverage, tea and crude drugs.

8. M. Paramasivam, et al [23], developed and reported a High-performance thin layer chromatographic method for quantitative determination of curcuminoids in *Curcuma longa* germplasm. Phytochemical investigation of the rhizomes of *Curcuma longa* leaded to the isolation of pharmacologically active curcuminoids viz Curcumin, Demethoxycurcumin and Bis-demethoxycurcumin. These were isolated from turmeric rhizomes by soxhlet extraction followed by column chromatography, crystallization and identified by spectroscopic studies. The purity of the curcuminoids was analysed by HPTLC method. The method employed TLC aluminium plates precoated with silica gel 60GF_{254} as the stationary phase. The solvent system consisted of chloroform: methanol (48:2, v/v). This system was found to give compact spots for Curcumin, Demethoxycurcumin and Bis-demethoxycurcumin (R_F value of 0.66 ± 0.02, 0.48 ± 0.02 and 0.30 ± 0.02), respectively. Densitometric analysis of curcuminoids was carried out in the absorption–reflection detection mode at 425 nm. Seven different germplasm of turmeric were analysed to detect the percentage of these three curcuminoids.

9. Wichitnithad W, et al [24], described a simple isocratic HPLC method for the simultaneous determination of curcuminoids in commercial turmeric extracts. The sample was prepared by dissolving the extract in acetonitrile and subsequently diluting with 50% acetonitrile. This solution was analyzed by reverse-phase chromatography on an Alltima C_{18} column with isocratic elution of Acetonitrile and 2% v/v acetic acid (40:60, v/v) at a flow rate of 2.0 mL/min, a column temperature of 33^0C and UV detection at 425 nm. The method was validated and applied for quantification of individual curcuminoids in commercial turmeric extracts. The method allowed simultaneous determination of Curcumin, Demethoxycurcumin and Bis-demethoxycurcumin in the concentration ranges of 10-60, 4-24 and 0.5-3.0 µg/mL, respectively. The limits of detection and quantification were 0.90 and 2.73 µg/mL, for Curcumin, 0.84 and 2.53 µg/mL, for Demethoxycurcumin and 0.08 and 0.23 µg/mL, for Bis-demethoxycurcumin and the percentage recoveries were 99.16-101.75 (%RSD < or = 1.11%), 99.50-101.01 (%RSD < or = 1.74%) and 99.67-101.92 (RSD < or = 1.31%).

10. B. k. Jadhav, et al [25], developed and validated a Isocratic Reversed Phase-HPLC Method for simultaneous determination of Curcumin, Demethoxycurcumin and Bis Demethoxycurcumin using RP C_{18} column. Mobile phase consisted of Acetonitrile: 0.1% trifluro-acetic acid (50:50) and flow rate was 1.5 ml/min and elution was monitored at 420 nm. Validation in selected condition showed that the chosen method was sensitive, selective, precise and reproducible with linear response of detector for the simultaneous determination of Curcumin (C), Demethoxycurcumin (DMC) and Bis-demethoxycurcumin (BDMC). The limits of detection were 27.99, 31.91, 21.81 ng/ml for C, DMC and BDMC, respectively. Linear range was from 100 to 600 ng/ml, the mean ± SD percent recoveries of curcuminoids were 99.87± 0.34, 100.09± 0.48 and 100.10 ± 0.60% of C, DMC and BDMC, respectively. Further the method was used for quantification of curcuminoids from turmeric rhizome.

11. Li J, Jiang Y, et al [26], developed a rapid and simple HPLC method for the determination of Curcumin in rat plasma: assay development, validation and application to a pharmacokinetic study of curcumin liposome, Curcumin and the IS emodin were separated on a Diamonsil C_{18} analytical column (4.6 x 100 mm, 5 microm) using Acetonitrile :5% acetic acid (75:25, v/v) as mobile phase at a flow rate

of 1.0 mL/min. The method was sensitive with a lower limit of quantitation of 1 ng/mL, with good linearity (r (2) >or= 0.999) over the linear range 1-500 ng/mL. The assay method was successfully applied to the study of the pharmacokinetics of Curcumin liposome in rats.

12. Yadav Vivek Ramshankar, et al [27], developed and reported a sensitive Reversed Phase HPLC Method for the determination of Curcumin. Chromatographic separation was achieved by using Merck C_{15} (250 cm X 4.6 mm) Column with mobile phase acetonitril: tetrahydrofuran: 2% acetic acid (50:30:20). The flow rate was 0.7 ml/min. The retention time was 4.587 minutes. The limit of detection and limit of quantification of Curcumin were between 3.68 to 8.125ng/ml for 50µL injection volumes. The percentage recovery of Curcumin was found to be 97.2 to 98.4 and relative standard deviation was 0.345 % and 1.160 %. The developed HPLC method could be therefore be applied to both in vitro studies of Curcumin formulations as well as drug estimation in biological samples.

13. Rubesh Kumar S et al [28], reported a simultaneous spectrophotometric estimation of Curcuminoids and Gallic Acid in bulk drug and Ayurvedic Polyherbal tablet dosage Form, In the present study, an attempt had been made to develop a Spectrophotometric method for the simultaneous estimation of curcuminoids and gallic acid in commercially marketed ayurvedic polyherbal formulation Nisha Amalaki vatti. Simultaneous equations (Vierodt's method) were performed by UV/Visible Spectrophotometric method. Curcuminoids had absorbance maxima at about 427 nm and Gallic acid maxima at about 227nm in methanol. The linearity was obtained in the concentration range of 10-50 mcg/mL for both curcuminoids and Gallic acid. The results of the analysis were validated statistically and by the recovery studies as per ICH guidelines.

14. Archana A Bele, et al [29], developmed and validated a HPTLC method for estimation of Curcumin, Ellagic acid in Gel Formulation. Gel formulation was chromatographed on silica gel 60 F254 TLC plate using toluene: ethyl acetate: methanol: formic acid (2.5: 2.5: 0.2: 0.8) as mobile phase. The method was found to give compact spots for the drug (R_f=0.5 ellagic acid, R_f for Curcumin 0.6).

15. Niraj Vyas, et al [30], reported a HPTLC method for simultaneous estimation of Curcumin and piperine in their crude powder mixture and Ayurvedic formulation. The separation was performed on TLC aluminum plates precoated with silica gel G60 F_{254}. Good separation was achieved in the mobile phase of Chloroform: Methanol (9.6:0.4 v/v) at R_f = 0.57 and 0.82 for curcumin and piperine respectively. Densitometric scanning of both compounds was carried out at 373 nm. The calibration curves showed a good linear relationship with r = 0.9998 and 0.9986 for curcumin and piperine respectively.

16. Arun gantait, et al [31], developed and validated HPTLC method for estimation of Curcumin in turmeric powder. The HPTLC separation was performed on precoated aluminum backed HPTLC plates of 0.2 mm layer thickness up to 80 mm at temperature of 20 ± 4^0C with 10 min. of chamber saturation. Under this condition the retention factor (Rf) of Curcumin was 0.43 and the compound was quantified at its absorbance maxima 427 nm. The limit of quantification was found to be 49 ng and 148 ng per spot, respectively. The response of Curcumin was linear over the range of 0.8 µg to 1.3 µg per spot with correlation coefficient 0.99395 indicated good relationship b/w peak area versus concentration. Recovery values from 99.60 to 99.73% showed that the reliability and reproducibility of the method were excellent.

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4. AIM AND PLAN OF WORK

The drug analysis plays an important role in the development, manufacture and therapeutic use of drug. Most of the pharmaceutical industries do the quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specifications and to determine how much of each components are present in the final product.

From the literature survey, it was found that there are some analytical methods reported for Curcumin either individually (or) in combination with other drugs by HPLC, HPTLC in raw drug, herbal extract and turmeric powder. Still now, no RP-HPLC method is available for the estimation of Curcumin in pharmaceutical Dosage form. So an attempt is made to develop RP-HPLC method for the estimation of Curcumin in Poly Herbal formulation.

The plan of present Work is as fallows

Method Development

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Selection of separation method and agent

Validation of the developed method

The developed method should be validated as per ICH Guidelines by using the various validation parameters such as,

- Accuracy
- Precision
- Linearity and of detection (LOD) / Limit of quantitation (LOQ)
- Selectivity / Specify
- Robustness / ruggedness
- System suitability.

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5.1 MATERIALS AND INSTRUMENTS USED

Materials:

a) Curcumin standard :

Curcumin standard was brought from the **VEEDISH CHEMICALS**, pune.

b) Drug sample :

Diazen Tablets was formulated by **APEX LABORATORIES,** Herbal division, Alathure,kanchipuram(Dt),Tamilanadu.

c) chemicals and solvents used :

S. No	Name	Grade
1.	Tetrahydrofuron	Merck (HPLC Grade)
2.	Water	Millipore
3.	Citric acid	AR grade
4.	Methanol	HPLC grade
5.	Acetonitrile	HPLC grade

d) Instruments used :

S. No	EQUIPMENT	MANUFACTURE	MODEL
1	Electronic balance-1	Mettle Toledo	Classic plus D value -0.1 mg
2	Electronic balance-2	Mettle Toledo Excellence	Xs205 D value -0.01 mg
3	HPLC pump Uv visible detector Column C_{18} [150 mm X 4.6 mm, 5µ]	 waters waters phenomonex	 E 2695 2489 Phen -s
4	Sonicator	sonorex	Sonorex dig 10 p
5	pH meter	Elico	
6	Membrane filter	AXIVA, NewDelhi	SRP15, 0.45mcm .025 mcm
7	Tablet tester	Campabell	DHT-25c
8	Milli pore water	Milli -Q	Q-POD
9	Hot air oven	Lab serve	
10	HPTLC	CAMAG linomat 4	
11	HPTLC scanner	CAMAG TLC scanner-3	
12	Moisture analyzer	Metter Toledo	HR84 halogen

5.2 METHODOLOGY

ANALYTICAL METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

(1)Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography.

From literature survey, it was found that octadecyl silane column C_{18} could be appropriately used for the separation of Curcumin.

(2)Selection of Wavelength

The sensitivity of the HPLC depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for the drugs to be detected. The wavelength for measurement was selected as 425 nm from the absorption spectrum.

(3)Selection of Mobile phase

Different mixtures of mobile phase with different ratios were selected and their chromatograms were recorded, many trails were done on Curcumin.

Traial-1

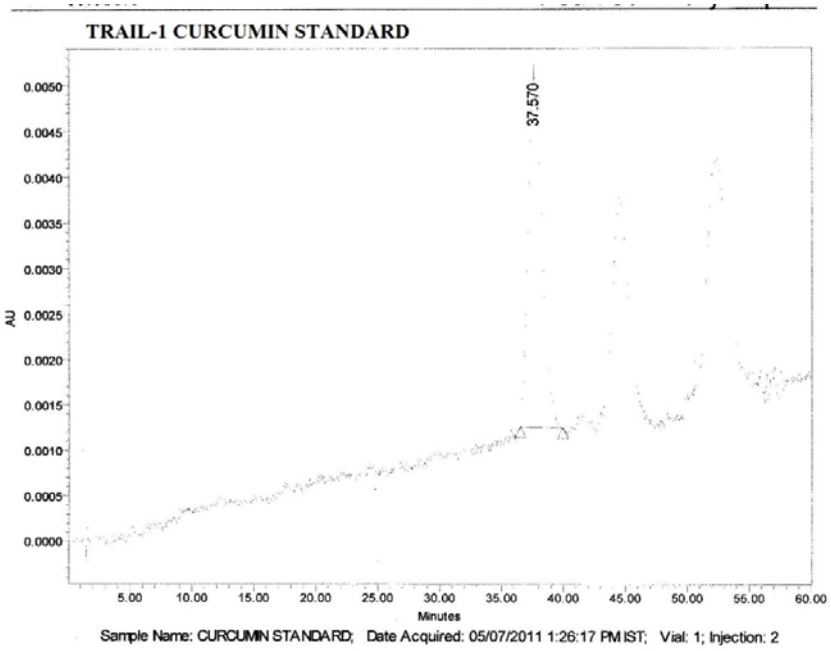
Preparation of mobile phase:

Measured accurately 12 ml of acetic acid and dissolved in 600 ml Millipore water. The mixed this solution to 400 ml of acetonitrile (ACN). pH was adjusted to 3.5 by using orthophosphoric acid and filtered through 0.45 µ m nylon membrane filter and degassed.

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 250 x 4.6mm x 5µ
Mobile phase	**:** Acetonitrile: 2% Acetic acid (400:600)
Flow rate	**:** 0.750 ml/min
Column temp	**:** 33^{o}C
Detection wavelength	**:** 425 nm
Run time	**:** 50 min
pH	**:** 3.5
Injection volume	**:** 20 µl

Chromatogram-1



Peak Summary with Statistics
Name:

	Sample Name	Vial	Inj	Retention Time (min)	Area	% Area	Height
1	CURCUMIN STANDARD	1	2	37.570	300600	100.00	3882
Mean				37.570			
Std. Dev.							
% RSD							

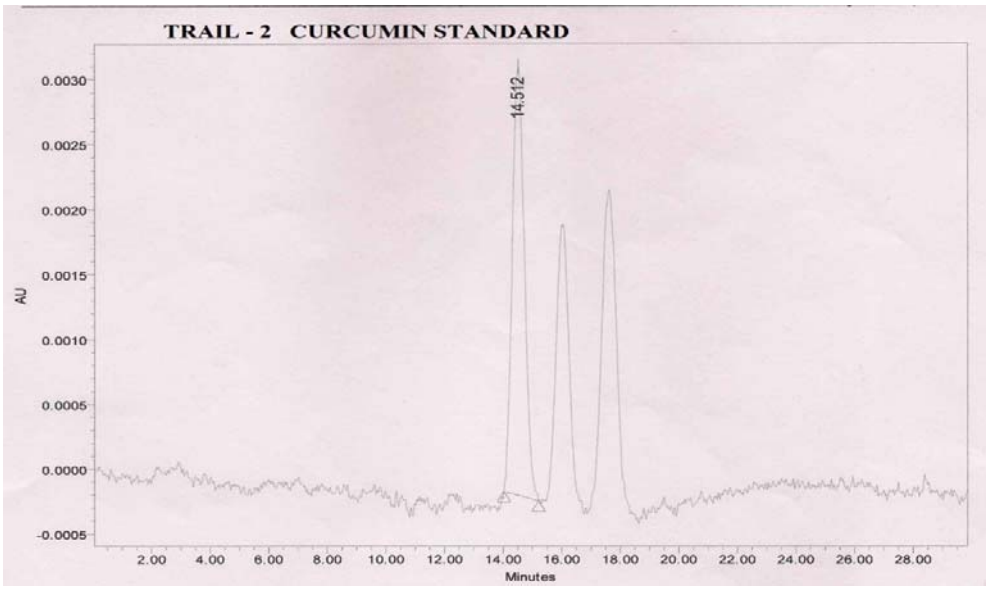
Observation: peak separation was not good, Rt was shifted to 35th minute and peak shape was not good.

Trail -2

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** Acetonitrile: 2% Acetic acid (400:600)
Flow rate	**:** 1 ml/min
Column temp	**:** 33^{o}C
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 4.2
Injection volume	: 20 µl

Chromagram-2



Peak Summary with Statistics
Name:

	Sample Name	Vial	Inj	Retention Time (min)	Area	% Area	Height
1	CURCUMIN STANDARD-1000PPM pH 4.2	1	1	14.512	95832	100.00	3300
Mean				14.512			
Std. Dev.							
% RSD							

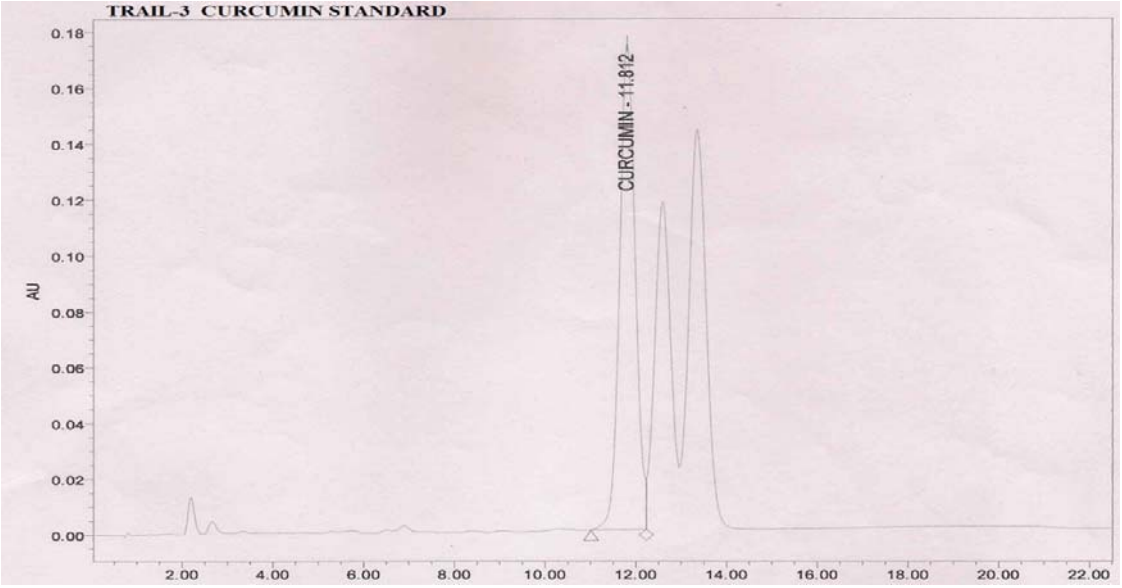
Observation: peak separation was not good and peak height was very low.

Trail -3

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** Acetonitrile: 2% Acetic acid (500:500)
Flow rate	**:** 1 ml/min
Column temp	**:** 44 ^{o}C
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl

Chromagram-3



Peak Summary with Statistics
Name: CURCUMIN

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	% Area	Height
1	std curcumin 500 ppm at 44c	2	1	CURCUMIN	11.812	4178859	100.00	173760
Mean					11.812			
Std. Dev.								
% RSD								

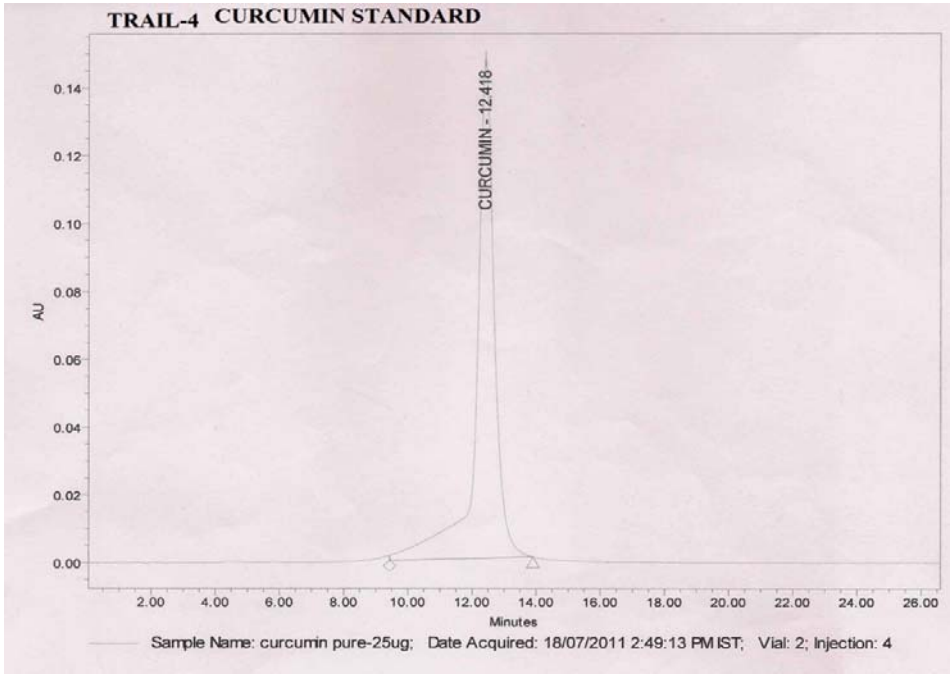
Observation: peaks were not separated.

Trail -4

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF: 1% citric Acid: ACN (300:600:100)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient Temp
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl

Chromagram-4



Peak Summary with Statistics
Name: CURCUMIN

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	% Area	Height
1	curcumin pure-25ug	2	4	CURCUMIN	12.418	5907772	100.00	146987
Mean					12.418			
Std. Dev.								
% RSD								

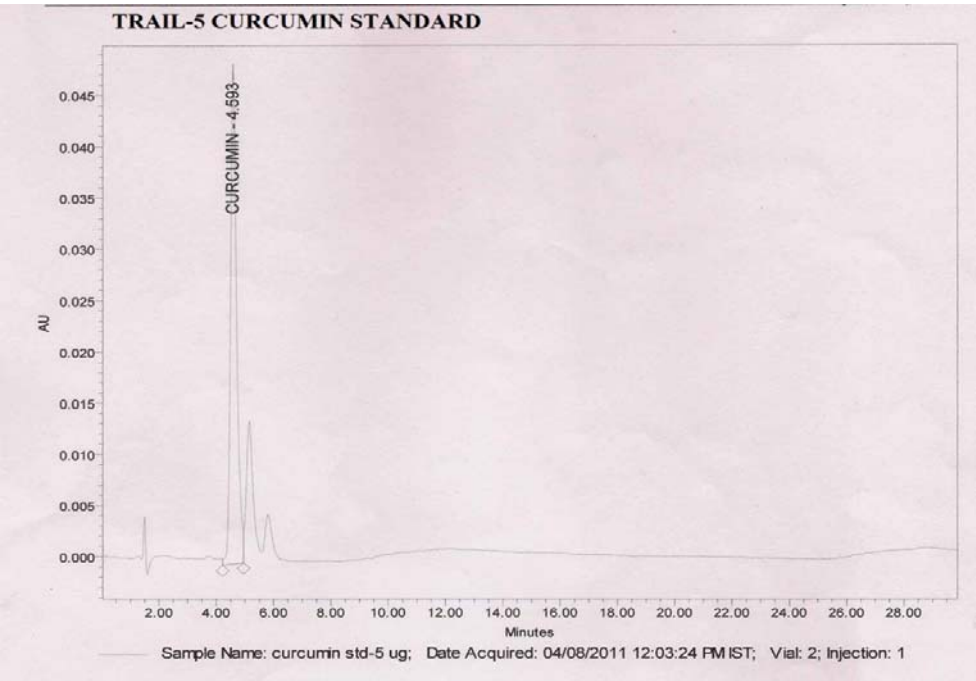
Observation: peak fronting was observed.

Trail -5

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF: 1% Citric acid (600:400)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl

Chromagram-5



Peak Summary with Statistics
Name: CURCUMIN

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	% Area	Height
1	curcumin std-5 ug	2	1	CURCUMIN	4.593	725528	100.00	48173
Mean					4.593			
Std. Dev.								
% RSD								

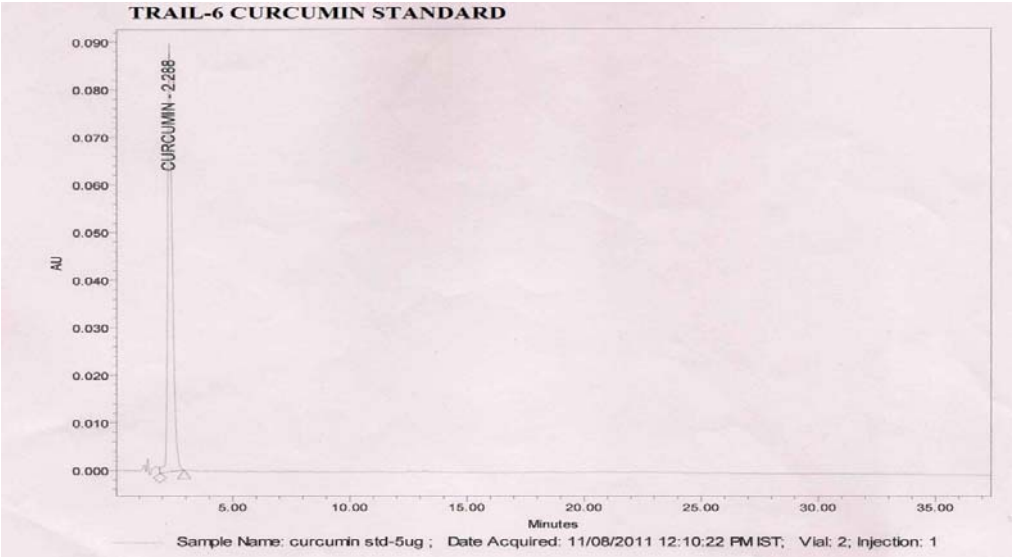
Observation: peaks were not separated

Trail -6

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF **:** 1% Citric acid (500:500)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl

Chromagram-6



Peak Summary with Statistics
Name: CURCUMIN

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	% Area	Height
1	curcumin std-5ug	2	1	CURCUMIN	2.288	1226934	100.00	88450
Mean					2.288			
Std. Dev.								
% RSD								

Observation: 3 peaks were merged in single peak.

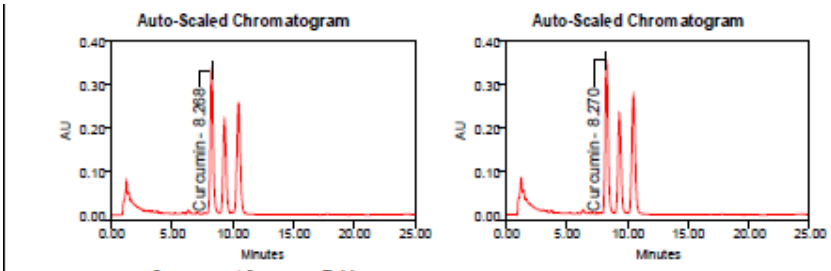
Optimized method:

Based on the above studies, the method development was carried out. Various proportions of Mobile phases were used in trails with the concentrations of solvents and various pH ranges (2-5) were tried with different flow rate (0.75 ml, 1.5 ml, 1 ml) and temperature (33 ^{0}C, 44 ^{0}C, 40 ^{0}C and ambient temp). Among these trails, following condition presented below had given the expected results

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF: 1% Citric acid (450:550)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl
Elution technique	**:** Isocratic technique

Chromagram-7



Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	ASSAY PREPARATION-1	Curcumin	8.268	5469658
2	ASSAY PREPARATION-1	Curcumin	8.270	5440343
Mean			8.269	5455000.5
Std. Dev.			0.001	20728.84
% RSD			0.0	0.4

Observation: peaks were well separated and RSD was passed.

ESTIMATION OF CURCUMIN IN POLY HERBAL FORMULATION

Procedure:

1) Preparation of mobile phase:

Accurately weighed 5.5 g of Citric acid and transferred into beaker containing 550 ml of Millipore water and Mixed correctly measured 450 ml of THF. Then shaked vigorously and filtered through the 0.45 µ vacuum filter and sonicated for 20 min.

Mobile phase ratio: 1% Citric acid: THF (550:450)

2) Preparation of blank:

Mobile phase was used as blank.

3) Preparation of Curcumin standard solution:

About 25 mg of Curcumin was accurately weighed and transferred into a 100 ml volumetric flask and added 25 ml of THF was added and sonicated for 15 min and volume was made up with Mobile phase. From the above stock solution 5ml was transferred to a 50 ml volumetric flask and the volume was made up with mobile phase. Filtered through the 0.45 µ membrane filter. (The final concentration of resulting was 25 µg/ml).

4) Preparation of sample solution:

20 tablets were weighed and average weight was found out. The tablets were powdered and about 5 mg equivalent of Curcumin (about 8.066 g of diazen tablet powder) was weighed and transferred into 200 ml volumetric flask. 40 ml THF was added and sonicated for 20 min and finally the volume was made up with mobile phase. Filtered through the 0.45 µ membrane filter. (The final concentration of resulting was 25 µg/ml).

5) Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF: 1% Citric acid (450:550)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient temperature
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl
Elution technique	**:** Isocratic technique

Procedure:

System was stabilized for 60 minutes with mobile phase and injected 20 µl of BLANK, STANDARD AND ASSAY preparations into the Chromatograph and measured the response of major peak.

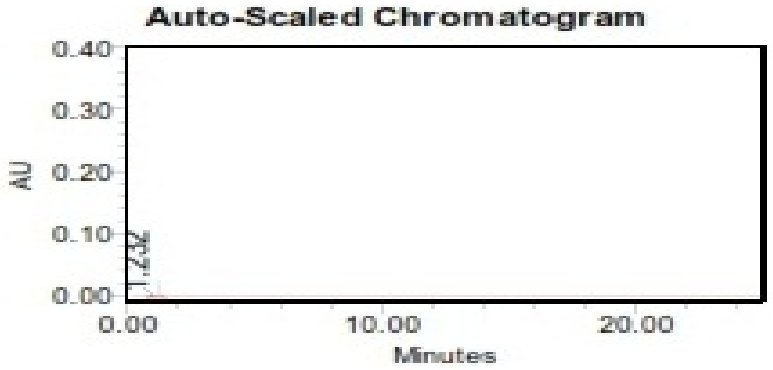
System suitability:

1. The tailing factor of Curcumin peak is not more than 2.0

2. The relative standard deviation for replicate injection is not more than 2.0%

3. The column efficiency as number of theoretical plates for Curcumin standard preparation should not be less than 2000.

Chromagram-7, Blank preparation



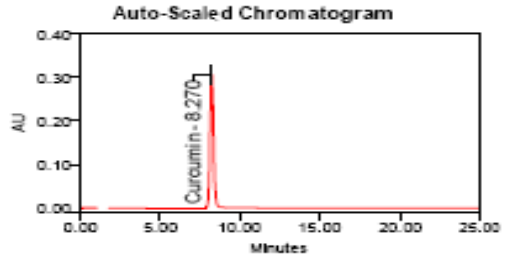
Chromagram-7, Curcumin standard preparation

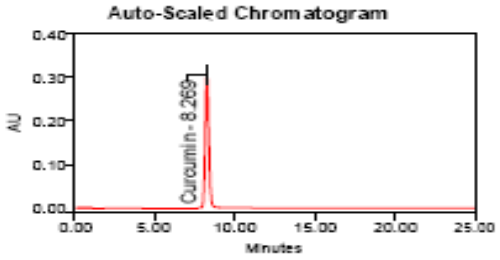
SAMPLE INFORMATION

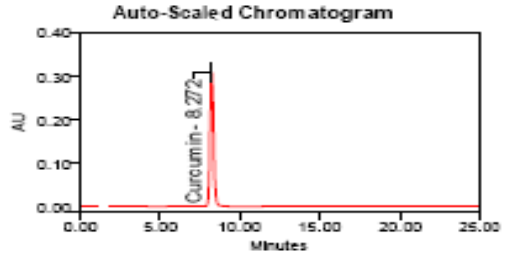
Sample Name:	CURCUMIN STANDARD	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		

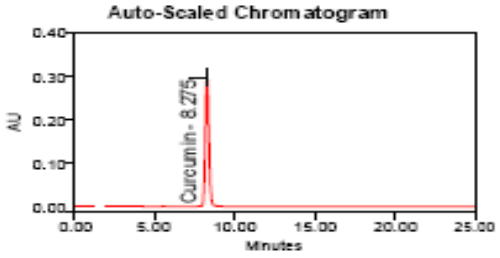
Date Acquired: 12/21/2011 1:43:12 PM IST, 12/21/2011 2:08:58 PM IST, 12/21/2011 2:34:45 PM IST, 12/21/2011

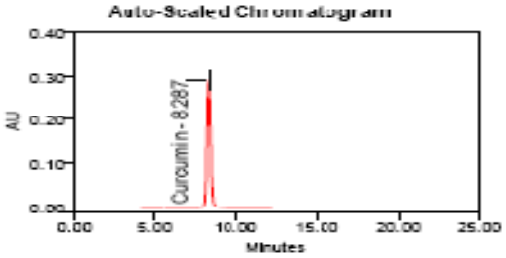
Date Processed: 1/5/2012 4:00:14 PM IST, 1/5/2012 4:00:23 PM IST, 1/5/2012 4:00:39 PM IST, 1/5/2012 4:00:50

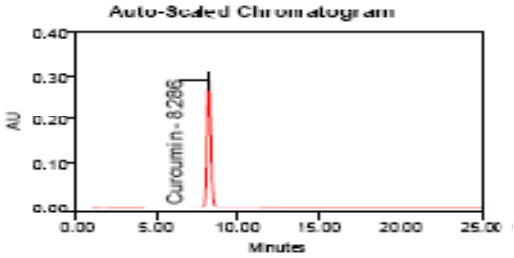












Component Summary Table
Name: Curcumin

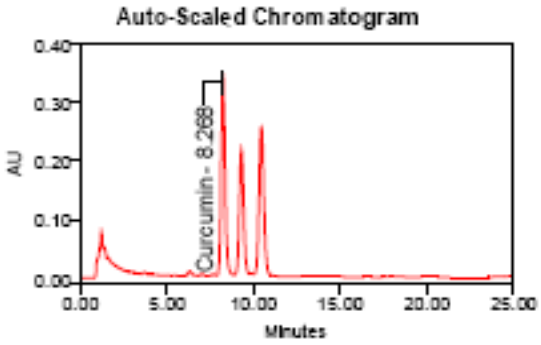
	Sample Name	Name	RT	Area
1	CURCUMIN STANDARD	Curcumin	8.270	4765464
2	CURCUMIN STANDARD	Curcumin	8.265	4769515
3	CURCUMIN STANDARD	Curcumin	8.272	4789437
4	CURCUMIN STANDARD	Curcumin	8.275	4629991
5	CURCUMIN STANDARD	Curcumin	8.287	4600278
6	CURCUMIN STANDARD	Curcumin	8.286	4600598
Mean			8.276	4692548.934
Std. Dev.			0.008	91115.356
% RSD			0.1	1.9

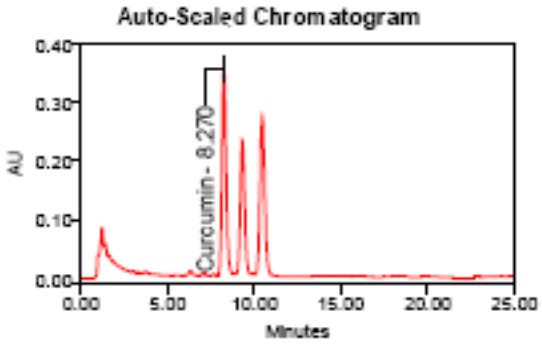
Chromagram-8,

Assay preparation

SAMPLE INFORMATION

Sample Name:	ASSAY PREPARATION-1	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	3	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/21/2011 4:18:15 PM IST, 12/21/2011 4:44:01 PM IST		
Date Processed:	1/5/2012 4:02:12 PM IST, 1/5/2012 4:02:54 PM IST		





	Sample name	Name	Retention Time(min)	Average area
1	Assay preparation -1	Curcumin	8.268	5455000.5
2	Assay preparation-2	Curcumin	8.294	5455603.5
3	Assay preparation-3	Curcumin	8.335	5432544.0
4	Assay preparation-4	Curcumin	8.403	5448002.0
5	Assay preparation-5	Curcumin	8.414	5454402.0
6	Assay preparation-6	Curcumin	8.433	5438293.0
% RSD			0.1	0.2

Calculation: The percentage of the labeled amount of Curcumin in the portion of tablet was calculated by the formula.

Formulation: Diazen tablet

Label claim: 0.294 mg of Curcumin and also contain 10% overage.

$$\frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{50} \times \frac{200}{WT} \times \frac{P}{100} \times AW$$

= ---------- mg of Curcumin per tablet

Where,

AS	:	Average peak area for standard preparation
AT	:	Average peak area for sample preparation
WS	:	Weight of Curcumin taken in mg for standard preparation
WT	:	Weight of tablets taken in mg
AW	:	Average weight of tablets
P	:	Purity of Curcumin working standard

ASSAY PREPARATION : 1

$$\frac{5455000.5}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8022} \times \frac{95}{100} \times 474.3$$

= 0.32256 mg of curcumin

= 109.713 % of curcumin

ASSAY PREPARATION : 2

$$\frac{5455603.5}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8057} \times \frac{95}{100} \times 474.3$$

= 0.321 mg of curcumin

= 109.249 % of curcumin

ASSAY PREPARATION : 3

$$\frac{5432544}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8043.4} \times \frac{95}{100} \times 474.3$$

= 0.320 mg of curcumin

= 108.971 % of curcumin

ASSAY PREPARATION : 4

$$\frac{5448002}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8024.3} \times \frac{95}{100} \times 474.3$$

= 0.322 mg of curcumin

= 109.541 % of curcumin

ASSAY PREPARATION : 5

$$\frac{5454402}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8064} \times \frac{95}{100} \times 474.3$$

= 0.321 mg of curcumin

= 109.13 % of curcumin

ASSAY PREPARATION : 6

$$\frac{5438293}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8064} \times \frac{95}{100} \times 474.3$$

= 0.320 mg of curcumin

= 108.808 % of curcumin

AVERAGE = **109.24 % of curcumin**

5.3 ANALYTICAL METHOD VALIDATION

After the development of RP-HPLC method for estimation of CURCUMIN in poly herbal formulation that method was validated by using following parameters

1) Parameters:

1.1 Specificity

1.2 Precision

a.Repeatability / method precision

b.Intermediate precision / ruggedness

1.3 Linearity & range

1.4 Accuracy / Recovery studies

1.5 System suitability

1.6 Robustness

2) Validation procedure:

2.1 Preparation of mobile phase:

Accurately weighed 5.5 g of Citric acid and transferred into beaker containing 550 ml of Millipore water and Mixed correctly measured 450 ml of THF. Then shaked vigorously and filtered through the 0.45 µ vacuum filter and sonicated for 20 min.

Mobile phase ratio: 1% Citric acid: THF (550:450)

2.2 Preparation of blank:

Mobile phase was used as blank.

2.3 Preparation of Curcumin standard solution:

About 25 mg of Curcumin was accurately weighed and transferred into a 100 ml volumetric flask and added 25 ml of THF and sonicated for 15 min then up the volume was made up with mobile phase. From the above stock solution 5 ml was transferred to a 50 ml volumetric flask and the volume was made up with mobile phase. Filtered through the 0.45 µ membrane filter. (The final concentration of resulting was 25 µg/ml).

2.4 Preparation of sample solution:

20 tablets were weighed and average weight was found out. The tablets were powdered, about 5 mg equivalent of Curcumin (about 8.066 g of diazen tablet powder) weighed and transferred into 200 ml volumetric flask, 40 ml THF was added and sonicated for 20 min. Finally the volume was made up with mobile phase. Filtered through the 0.45 µ membrane filter. (the final concentration of resulting was 25 µg/ml).

Chromatographic conditions:

Column	**:** symmetry $C_{18,}$ 150 x 4.6mm x 5µ
Mobile phase	**:** THF: 1% Citric acid (450:550)
Flow rate	**:** 1 ml/min
Column temp	**:** Ambient temperature
Detection wavelength	**:** 425 nm
Run time	**:** 25 min
pH	**:** 2.8
Injection volume	**:** 20 µl
Elution technique	**:** Isocratic technique

1.1 SPECIFICITY:

1.1.1 SELECTIVITY:

Preparation of analytical solutions

a.Blank **:** Mobile phase was used as blank

b.Standard preparation **:** prepared as described under **2.3.**

c.Assay preparation **:** prepared as described under **2.4.**

d.Placebo preparation **:** Transferred an accurately weighed quantity of about 8.06 g of placebo (diazen tablets excipient mixture) into a 200 ml volumetric flask and added 40 ml of the tetra hydro furan and sonicated for 20 minutes. The volume was made up with mobile phase. Filtered the resulting solution through 0.45 µm membrane filter.

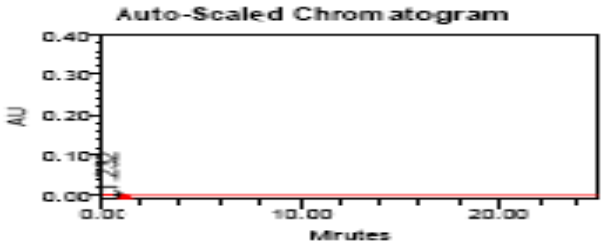
Table-1: Sequence for selectivity

S. No.	Sample name	Number of injections
1	Blank	1
2	Standard preparation	6
3	Blank for carry over	1
4	Placebo preparation	2
6	Assay preparation	2
7	Standard preparation (Closing)	1

1.1.2 Procedure: Separately injected 20 µl each of all the above solutions described under **1.1.1** into the chromatograph and continued the chromatography until the retention time of Curcumin peak. Measure the responses the peak for all the solutions. (Refer **Table-1** for sequence of injections).

1.1.3 Acceptance criteria: The interference between peaks obtained for all the solutions should be **NIL**. The peaks should well separate from the peaks obtained with blank, placebo, standard and test solution.

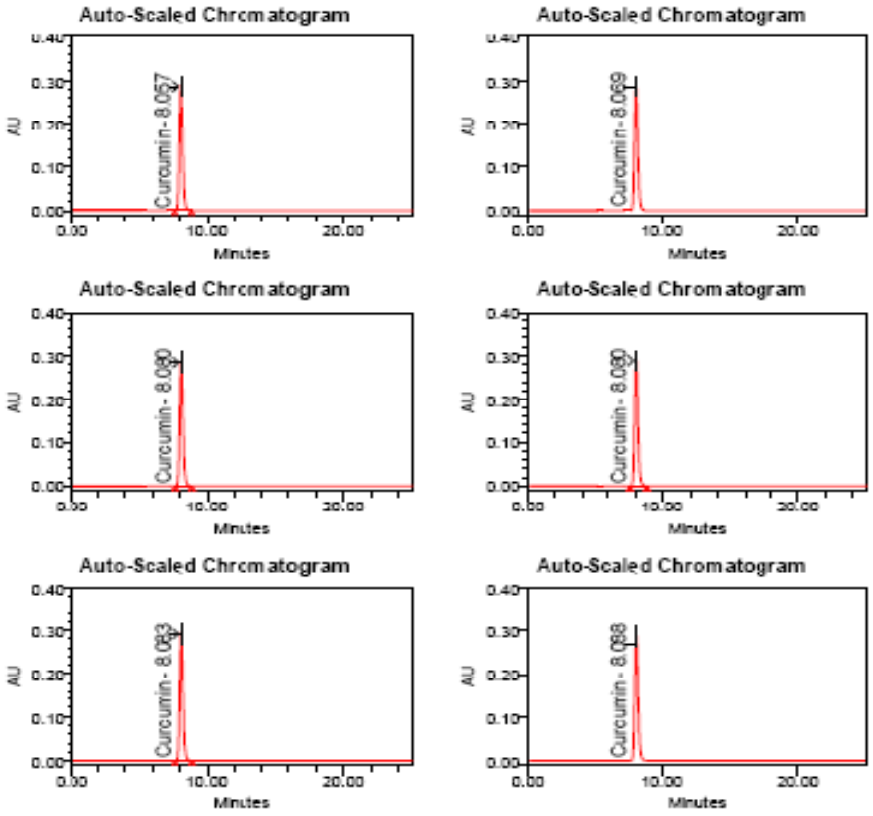
Chromagram-10 Blank preparation



Chromagram-11 Standard preparation

SAMPLE INFORMATION

Sample Name:	CURCUMIN STANDARD	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 1:11:26 PM IST, 12/17/2011 1:37:11 PM IST, 12/17/2011 2:02:57 PM IST, 12/17/2011		
Date Processed:	12/19/2011 12:24:41 PM IST, 12/19/2011 12:24:46 PM IST, 12/19/2011 12:24:50 PM IST,		



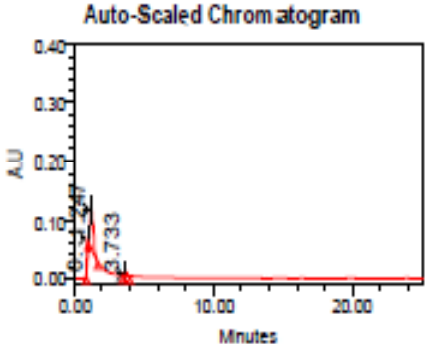
Component Summary Table
Name: Curcumin

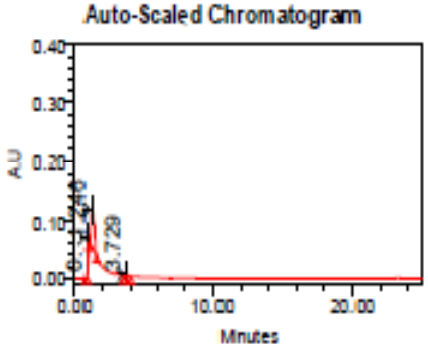
	Sample Name	Name	RT	Area
1	CURCUMIN STANDARD	Curcumin	8.057	4801791
2	CURCUMIN STANDARD	Curcumin	8.069	4806736
3	CURCUMIN STANDARD	Curcumin	8.080	4816578
4	CURCUMIN STANDARD	Curcumin	8.080	4823163
5	CURCUMIN STANDARD	Curcumin	8.083	4847330
6	CURCUMIN STANDARD	Curcumin	8.088	4861349
Mean			8.076	4826157.894
Std. Dev.			0.011	23487.867
% RSD			0.1	0.5

Chromagram-12 placebo preparation

SAMPLE INFORMATION

Sample Name:	PLACEBO PREPARATION	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	3	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 4:11:49 PM IST, 12/17/2011 4:37:35 PM IST		
Date Processed:	12/19/2011 12:26:31 PM IST, 12/19/2011 12:26:35 PM IST		





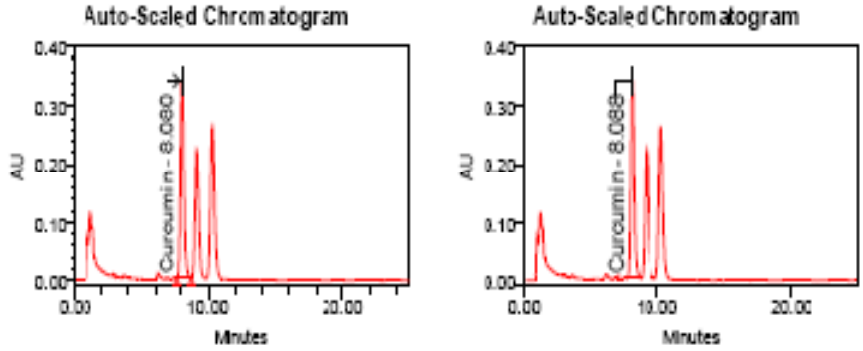
Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	PLACEBO PREPARATION	Curcumin	8.066	
2	PLACEBO PREPARATION	Curcumin	8.066	
Mean			8.066	
Std. Dev.			0.000	
% RSD			0.0	

Chromagram-13 Assay Preparation

SAMPLE INFORMATION

Sample Name:	ASSAY PREPARATION	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	4	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 5:03:23 PM IST, 12/17/2011 5:29:05 PM IST		
Date Processed:	12/19/2011 12:26:57 PM IST, 12/19/2011 12:27:07 PM IST		



Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	ASSAY PREPARATION	Curcumin	8.080	5868697
2	ASSAY PREPARATION	Curcumin	8.088	5870489
Mean			8.084	5869593.068
Std. Dev.			0.006	1267.568
% RSD			0.1	0.0

1.2) PRECISION

Objective: Establish the precision of the analytical method using the two methods. .

A) Repeatability: Establish the repeatability of the analytical method by estimating the assay for six different sample preparations of same batch. Calculate percentage content for all six-sample preparations and the %RSD was reported for the same.

B) Intermediate precision (Ruggedness): Establish the ruggedness of the analytical method by estimating the assay for six different sample preparations of same batch by a different analyst using a different HPLC system with different lot of column on a different day. Calculate percentage content for all six-sample preparations and report the %RSD for the same.

A) REPEATABILITY

a) Blank: Mobile phase was used as blank

b) Standard preparation: prepared as described under **2.3.**

c) Assay preparation : prepare as described under **2.4.**

S. No.	Sample name	Number of injections
1	Blank	1
2	Standard preparation	6
3	Assay preparation-1	2
4	Assay preparation-2	2
5	Assay preparation-3	2
6	Standard preparation (Bracketing)	1
7	Assay preparation-4	2
8	Assay preparation-5	2
9	Assay preparation-6	2
10	Standard preparation (Closing)	1

Procedure: Separately injected 20 µl of standard preparation in replicate and assay preparations in duplicate into the chromatograph and measure the peak responses for the major peak. The percentage of the labeled amount of Curcumin in the portion of tablets was calculated by the formula.

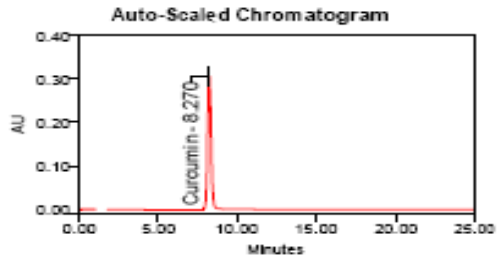
Chromagram-16 standard preparation for repeatability

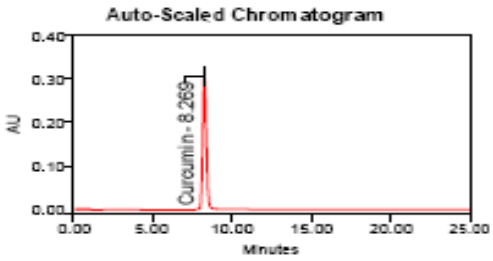
SAMPLE INFORMATION

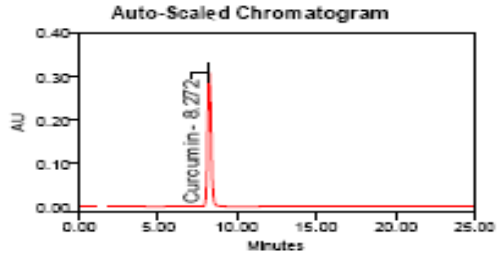
Sample Name:	CURCUMIN STANDARD	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		

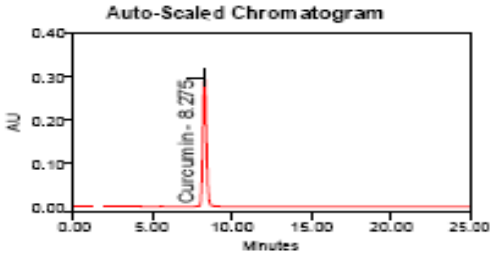
Date Acquired: 12/21/2011 1:43:12 PM IST, 12/21/2011 2:08:58 PM IST, 12/21/2011 2:34:45 PM IST, 12/21/2011

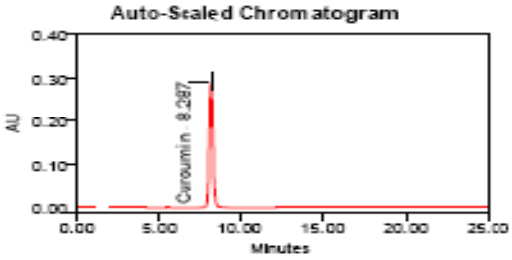
Date Processed: 1/5/2012 4:00:14 PM IST, 1/5/2012 4:00:23 PM IST, 1/5/2012 4:00:39 PM IST, 1/5/2012 4:00:50

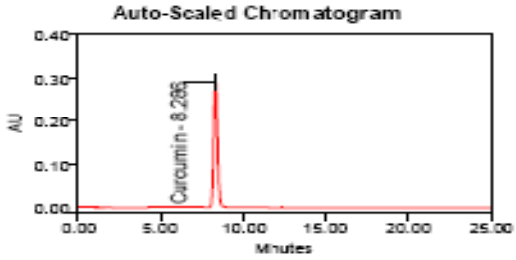












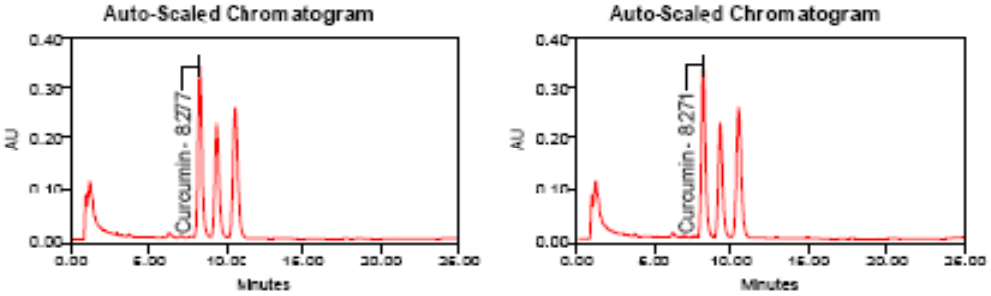
Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	CURCUMINSTANDARD	Curcumin	8.270	4765464
2	CURCUMINSTANDARD	Curcumin	8.269	4769515
3	CURCUMINSTANDARD	Curcumin	8.272	4789437
4	CURCUMINSTANDARD	Curcumin	8.275	4629991
5	CURCUMINSTANDARD	Curcumin	8.287	4600278
6	CURCUMINSTANDARD	Curcumin	8.286	4600598
Mean			8.276	4692546.924
Std. Dev.			0.008	91115.356
% RSD			0.1	1.9

Chromagram-17 Assay preparation for repeatability

SAMPLE INFORMATION

Sample Name:	ASSAY PREPARATION-6	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	9	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/20/2011 10:15:10 PM IST, 12/20/2011 10:40:54 PM IST		
Date Processed:	12/21/2011 12:02:01 PM IST, 12/21/2011 12:02:07 PM IST		



	Name of the sample	name	RT	Average area
1	Assay preparation-1	Curcumin	8.249	5557503.0
2	Assay preparation-2	Curcumin	8.263	5556437.0
3	Assay preparation-3	Curcumin	8.310	5530057.0
4	Assay preparation-4	Curcumin	8.300	5574890.0
5	Assay preparation-5	Curcumin	8.290	5575590.0
6	Assay preparation-6	Curcumin	8.274	5532288.0
%RSD			0.1	0.3

Calculation:

$$\frac{AT}{AS} \times \frac{WS}{100} X \frac{5}{50} \times \frac{200}{WT} \times \frac{P}{100} \times AW$$

$$= \text{---------- mg of Curcumin per tablet.}$$

PRECISION

REPEATABILITY

$$\frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{50} \times \frac{200}{WT} \times \frac{\text{Purity of std}}{100} \times \text{AVG WT}$$

ASSAY PREPARATION : 1

$$\frac{5557503}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8072.6} \times \frac{95}{100} \times 474.3$$

= 0.3222 mg of curcumin

= 109.6 % of curcumin

ASSAY PREPARATION : 2

$$\frac{5556437}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8082.6} \times \frac{95}{100} \times 474.3$$

= 0.322 mg of curcumin

= 109.44 % of curcumin

ASSAY PREPARATION : 3

$$\frac{5530057}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8091} \times \frac{95}{100} \times 474.3$$

= 0.320 mg of curcumin

= 108.81 % of curcumin

ASSAY PREPARATION : 4

$$\frac{5574890}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8081.6} \times \frac{95}{100} \times 474.3$$

= 0.323 mg of curcumin

= 109.82 % of curcumin

ASSAY PREPARATION : 5

$$\frac{5575590}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8083.1} \times \frac{95}{100} \times 474.3$$

= 0.323 mg of curcumin

= 109.81 % of curcumin

ASSAY PREPARATION : 6

$$\frac{5532288}{4736503} \times \frac{24.6}{100} \times \frac{5}{50} \times \frac{200}{8092.1} \times \frac{95}{100} \times 474.3$$

= 0.320 mg of curcumin

= 108.84 % of curcumin

AVERAGE = 109.39 % of curcumin

B) INTERMEDIATE PRECISION (RUGGEDNESS)

Raggedness was done by different analyst using a different HPLC system with a different lot of column on a different day should carried out this experiment.

a) Blank: Mobile phase was used as blank.

b) Standard preparation: Prepare as described under **2.3**.

c) Assay preparation: Prepare as described under **2.4**

d) Table-4: Intermediate precision / Ruggedness sequence for curcumin Tablets

S. No.	Sample name	Number of injections
1	Blank	1
2	Standard preparation	6
3	Assay preparation-1	2
4	Assay preparation-2	2
5	Assay preparation-3	2
6	Standard preparation (Bracketing)	1
7	Assay preparation-4	2
8	Assay preparation-5	2
9	Assay preparation-6	2
10	Standard preparation (Closing)	1

Procedure: Separately injected 20 µl of standard preparation in replicate and assay preparations in duplicate into the chromatograph and measured the peak responses for the major peak. The percentage of the labeled amount of diazen tablet was calculated

Acceptance criteria

1.2.2 for repeatability

RSD of the assay of all six-sample preparations of same batch should not be more than 2.0%.

1.2.1 For intermediate precision (Ruggedness)

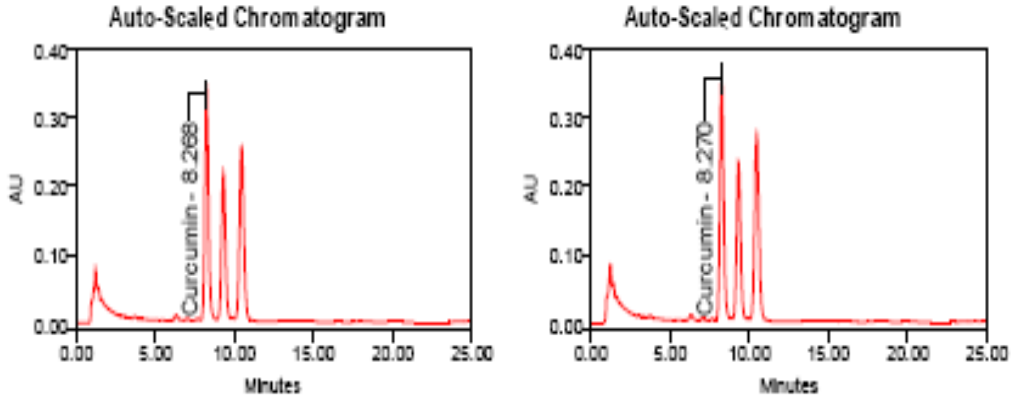
a) The relative standard deviation for the assay values of six sample preparations of same batch should not be more than 2.0%.

b) The difference in the assay of same batch between two analysts should not be more than 5.0%.

Chromagram-18 Assay preparation for intermediate precision

SAMPLE INFORMATION

Sample Name:	ASSAY PREPARATION-1	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	3	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/21/2011 4:18:15 PM IST, 12/21/2011 4:44:01 PM IST		
Date Processed:	1/5/2012 4:02:12 PM IST, 1/5/2012 4:02:54 PM IST		



	Sample name	Name	Retention Time(min)	Average area
1	Assay preparation -1	Curcumin	8.268	5455000.5
2	Assay preparation-2	Curcumin	8.294	5455603.5
3	Assay preparation-3	Curcumin	8.335	5432544.0
4	Assay preparation-4	Curcumin	8.403	5448002.0
5	Assay preparation-5	Curcumin	8.414	5454402.0
6	Assay preparation-6	Curcumin	8.433	5438293.0
% RSD			0.1	0.2

PRECISION

INTERMEDIATE PRECISION:

$$\frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{50} \times \frac{200}{WT} \times \frac{\text{Purity of std}}{100} \times \text{AVG WT}$$

ASSAY PREPARATION : 1

$$\frac{5455000.5}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8022} \times \frac{95}{100} \times 474.3$$

= **0.32256 mg of curcumin**

= **109.713 % of curcumin**

ASSAY PREPARATION : 2

$$\frac{5455603.5}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8057} \times \frac{95}{100} \times 474.3$$

= **0.321 mg of curcumin**

= **109.249 % of curcumin**

ASSAY PREPARATION : 3

$$\frac{5432544}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8043.4} \times \frac{95}{100} \times 474.3$$

= **0.320 mg of curcumin**

= **108.971 % of curcumin**

ASSAY PREPARATION : 4

$$\frac{5448002}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8024.3} \times \frac{95}{100} \times 474.3$$

= **0.322 mg of curcumin**

= **109.541 % of curcumin**

ASSAY PREPARATION : 5

$$\frac{5454402}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8064} \times \frac{95}{100} \times 474.3$$

= **0.321 mg of curcumin**

= **109.13 % of curcumin**

ASSAY PREPARATION : 6

$$\frac{5438293}{4692546.9} \times \frac{24.7}{100} \times \frac{5}{50} \times \frac{200}{8064} \times \frac{95}{100} \times 474.3$$

= **0.320 mg of curcumin**

= **108.808 % of curcumin**

AVERAGE = **109.24 % of curcumin**

1.3) LINEARITY & RANGE

Objective: Using the following two methods the linearity of the analytical assay was established.

Linearity & range of Curcumin in standard preparations from of 80% to 120% of test concentration: Demonstrate the linearity of the analytical method for assay by injecting the various concentrations of standard preparation prepared in the range of 80% to 120%, into the chromatograph, covering 5 different concentrations. A plot between the Concentrations in ppm vs peak response of Curcumin was constructed. The slope, intercept and regression coefficient from the plot obtained for concentration vs. Peak response of Curcumin in standard preparation were reported.

1.3.1. Preparation of analytical solutions for linearity & range of Curcumin in standard preparation:

a) **Blank:** Mobile phase was used as blank**.**
b) **Standard preparation:** Prepare as described under **2.3**.
c) **Stock standard preparation:** Transferred an accurately weighed quantity of about 25 mg of Curcumin working standard into a 100 ml volumetric flask. 25ml of tetra hydro furan was added and sonicated to dissolve and the volume was made up with mobile phase.
d) **80% linearity solution:** 4.0ml of standard stock solution was pipetted out in 50ml volumetric flask and diluted to volume with mobile phase. Filtered the solution through 0.45µm membrane filter.
e) **90% linearity solution:** 4.5 ml of standard stock solution was pipetted out in 50ml volumetric flask and diluted to volume with mobile phase. Filtered the solution through 0.45µm membrane filter.
f) **100% linearity solution:** 5.0ml of standard stock solution was pipetted out in 50ml volumetric flask and diluted to volume with mobile phase. Filtered the solution through 0.45µm membrane filter**.**
g) **110% linearity solution:** 5.5ml of standard stock solution was pipetted out in 50ml volumetric flask and diluted to volume with mobile phase. Filtered the solution through 0.45µm membrane filter.

h) **120% linearity solution:** 6.0ml of standard stock solution was pipetted out in 50ml volumetric flask and diluted to volume with mobile phase. Filtered the solution through 0.45µm membrane filter.

Table-5: Linearity sequence

S. No.	Sample name	Number of injections
1	Blank	1
2	Standard preparation	6
3	80% linearity solution	3
4	90% linearity solution	3
5	Standard preparation (Bracketing)	1
6	100% linearity solution	3
7	110% linearity solution	3
8	120% linearity solution	3
9	Standard preparation (Closing)	1

1.3.1 Procedure: Separately injected 20 µl of standard preparation in replicate into the chromatograph and measure the peak responses for the major peak.RSD for the 6 replicate injections was calculated. Continued the injections of 20µl in triplicate for all preparations described above into the chromatograph and the chromatograms were recorded. Measured the peak responses for the major peak for all the solutions. A plot between the concentrations vs the average peak responses of Curcumin was drawn. The slope, intercept and regression coefficient from the plot were calculated.

1.3.2 Calculations: A plot of concentrations vs the average peak responses for Curcumin standard solutions was drawn and the slope, intercept and regression coefficient from the plot obtained were reported.

1.3.3 Acceptance Criteria: The relationship between the concentration and response of Curcumin should be linear in the specified range and the regression coefficient should not be less than 0.995.

Chromagram-19 Curcumin standard for linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN STANDARD	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/16/2011 4:54:52 PM IST, 12/16/2011 5:20:38 PM IST, 12/16/2011 5:46:24 PM IST, 12/16/2011		
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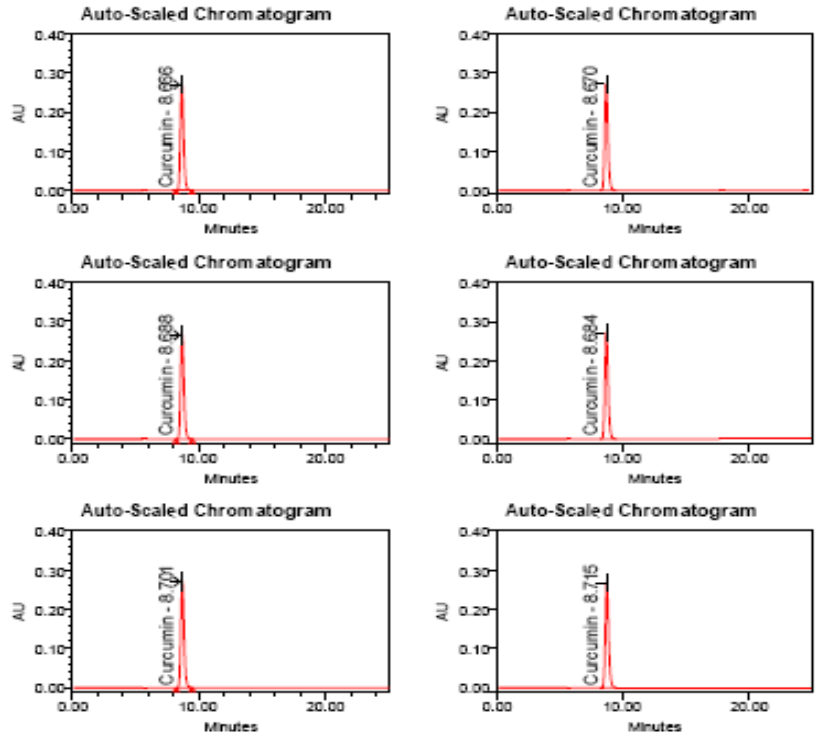


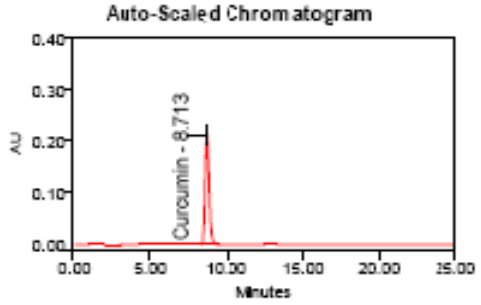
Component Summary Table
Name: Curcumin

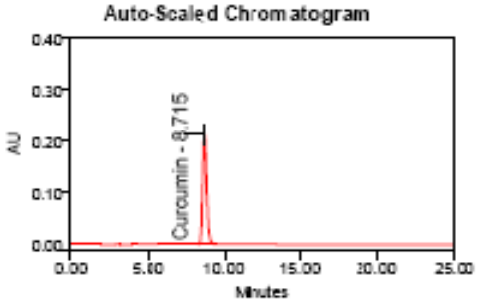
	Sample Name	Name	RT	Area
1	CURCUMIN STANDARD	Curcumin	8.666	4766585
2	CURCUMIN STANDARD	Curcumin	8.670	4773300
3	CURCUMIN STANDARD	Curcumin	8.688	4760647
4	CURCUMIN STANDARD	Curcumin	8.684	4782607
5	CURCUMIN STANDARD	Curcumin	8.701	4798006
6	CURCUMIN STANDARD	Curcumin	8.715	4802403
Mean			8.687	4780591.243
Std. Dev.			0.019	16912.666
% RSD			0.2	0.4

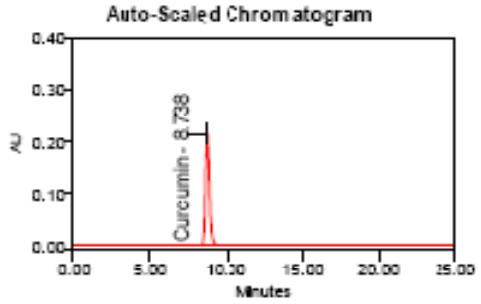
Chromate gram- 20 Curcumin 80% linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN 80% LINEARITY	Acquired By:	Rav
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSE
Vial:	3	Processing Method:	Curcumin_PM
injection #:	1, 2, 3	Channel Name:	W2489 ChA
injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/16/2011 7:29:25 PM IST, 12/16/2011 7:55:13 PM IST, 12/16/2011 8:20:57 PM IST		
Date Processed:	12/17/2011 4:12:27 PM IST, 12/17/2011 4:12:32 PM IST, 12/17/2011 4:12:40 PM IST		







Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	CURCUMIN 80% LINEARITY SOLUTION	Curcumin	8.713	3819414
2	CURCUMIN 80% LINEARITY SOLUTION	Curcumin	8.715	3813191
3	CURCUMIN 80% LINEARITY SOLUTION	Curcumin	8.738	3820567
Mean			8.722	3817757.465

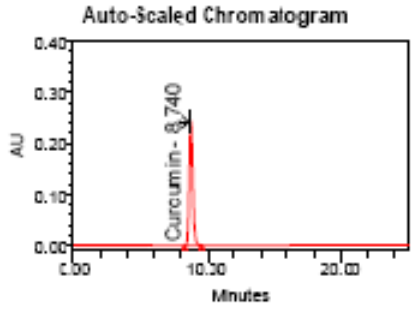
Component Summary Table
Name: Curcumin

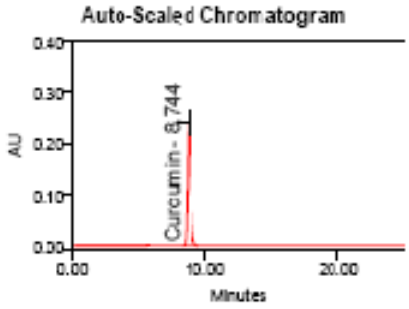
	Sample Name	Name	RT	Area
Std. Dev.			0.014	4003.796
% RSD			0.2	0.1

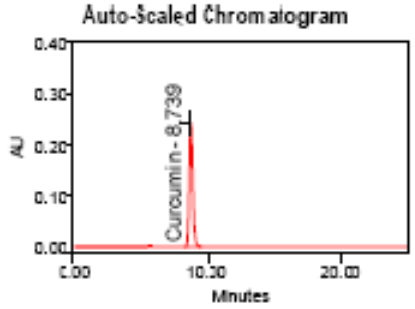
Chromatogram- 21 Curcumin 90% linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN 90% LINEARITY	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	4	Processing Method:	Curcumin_PM
Injection #:	1 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/16/2011 8:46:44 PM IST, 12/16/2011 9:12:31 PM IST, 12/16/2011 9:38:17 PM IST		
Date Processed:	12/17/2011 4:12:56 PM IST, 12/17/2011 4:12:59 PM IST, 12/17/2011 4:13:01 PM IST		







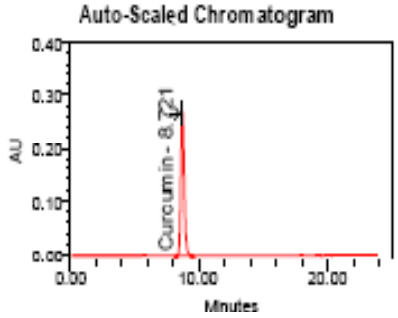
Component Summary Table
Name: Curcumin

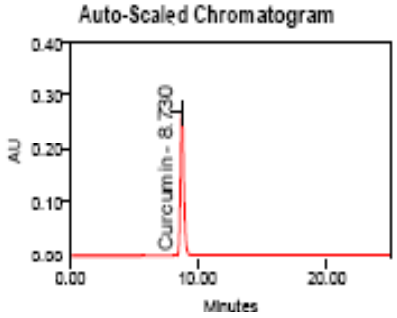
	Sample Name	Name	RT	Area
1	CURCUMIN 90% LINEARITY SOLUTION	Curcumin	8.740	4354053
2	CURCUMIN 90% LINEARITY SOLUTION	Curcumin	8.739	4351780
3	CURCUMIN 90% LINEARITY SOLUTION	Curcumin	8.744	4343725
Mean			8.741	4349852.344
Std. Dev.			0.003	5427.094
% RSD			0.1	0.1

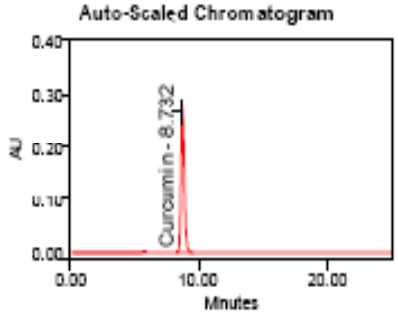
Chromatogram -22 Curcumin 100% linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN 100% LINEARITY	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MDLT
Vial:	5	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/16/2011 10:29:53 PM IST, 12/16/2011 10:55:38 PM IST, 12/16/2011 11:21:24 PM IST		
Date Processed:	12/17/2011 4:13:15 PM IST, 12/17/2011 4:13:18 PM IST, 12/17/2011 4:13:20 PM IST		







Component Summary Table

Name: Curcumin

	Sample Name	Name	RT	Area
1	CURCUMIN 100% LINEARITY SOLUTION	Curcumin	8.721	4808335
2	CURCUMIN 100% LINEARITY SOLUTION	Curcumin	8.730	4814405
3	CURCUMIN 100% LINEARITY SOLUTION	Curcumin	8.732	4813591
Mean			8.728	4812110.706
Std. Dev.			0.006	3294.703
% RSD			0.1	0.1

Chromatogram -23 Curcumin 110% linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN 110% LINEARITY	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	6	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/16/2011 11:47:08 PM IST, 12/17/2011 12:12:53 AM IST, 12/17/2011 12:38:39 AM IST		
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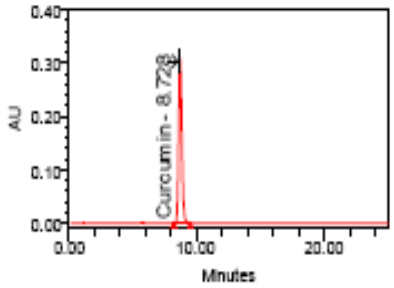
Auto-Scaled Chromatogram



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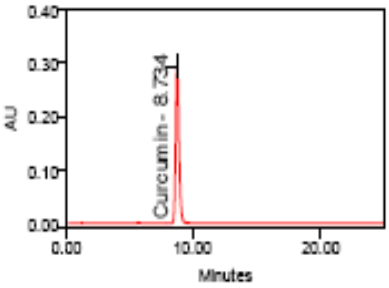
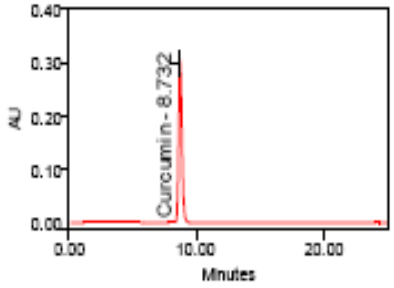


Auto-Scaled Chromatogram



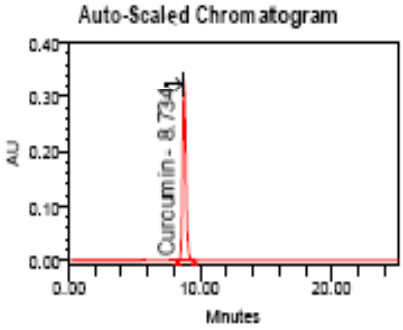
Component Summary Table
Name: Curcumin

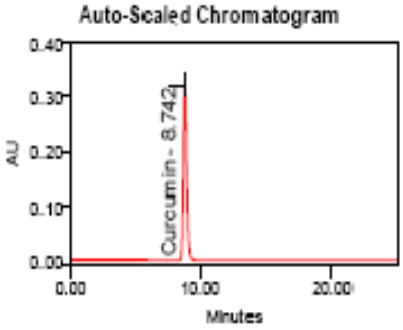
	Sample Name	Name	RT	Area
1	CURCUMIN 110% LINEARITY SOLUTION	Curcumin	8.728	5356732
2	CURCUMIN 110% LINEARITY SOLUTION	Curcumin	8.734	5357370
3	CURCUMIN 110% LINEARITY SOLUTION	Curcumin	8.732	5364663
Mean			8.731	5359588.084
Std. Dev.			0.003	4406.250
% RSD			0.0	0.1

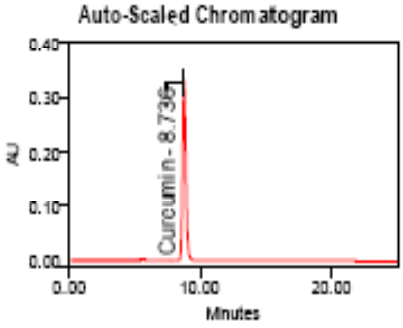
Chromatogram -24 Curcumin 120% linearity

SAMPLE INFORMATION

Sample Name:	CURCUMIN 120% LINEARITY	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	7	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 1:04:22 AM IST, 12/17/2011 1:30:09 AM IST, 12/17/2011 1:55:52 AM IST		
Date Processed:	12/17/2011 4:14:07 PM IST, 12/17/2011 4:14:09 PM IST, 12/17/2011 4:14:11 PM IST		







Component Summary Table
Name: Curcumin

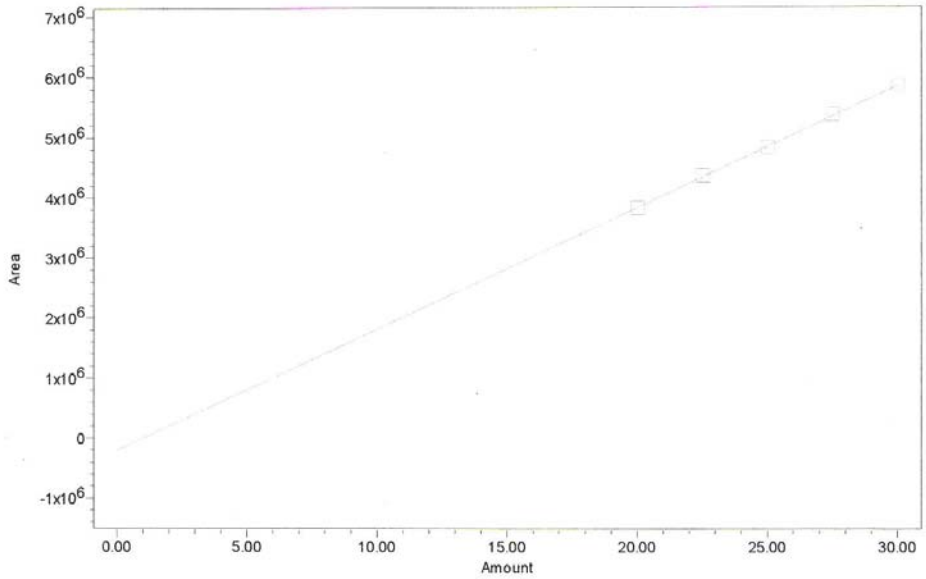
	Sample Name	Name	RT	Area
1	CURCUMIN 120% LINEARITY SOLUTION	Curcumin	8.734	5826799
2	CURCUMIN 120% LINEARITY SOLUTION	Curcumin	8.742	5826317
3	CURCUMIN 120% LINEARITY SOLUTION	Curcumin	8.736	5844209
Mean			8.738	5832441.505
Std. Dev.			0.004	10193.535
% RSD			0.0	0.2

Empower 2 software

LINEARITY REPORT

Processing Method:	Curcumin_PM	System:	GPD_AD001
Processing Method ID:	1149	Channel:	W2489 ChA
Calibration ID:	1142	Proc. Chnl. Descr.:	****
Date Calibrated:	12/17/2011 4:20:28 PM IST		

Calibration Plot



Name: Curcumin; Processing Method: Curcumin_PM; Fit Type: Linear (1st Order); Cal Curve Id: 1143;
A: -2.047538e+005; B: 2.015642e+005; C: 0.000000e+000; D: 0.000000e+000; R^2: 0.999410

Peak: Curcumin

	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	80%	20.000000	3817757.460000	19.956481	-0.218	Yes	No
2	90%	22.500000	4349852.340000	22.596310	0.428	Yes	No
3	100%	25.000000	4812110.700000	24.889666	-0.441	Yes	No
4	110%	27.500000	5359588.080000	27.605811	0.385	Yes	No
5	120%	30.000000	5832441.500000	29.951731	-0.161	Yes	No

1.4 ACCURACY

1.4.1 The accuracy of the analytical method was calculated by using the following method.

Recovery experiments: Performed the recovery studies by adding known quantities of Curcumin working standard to known quantity of placebo (diazen Tablets excipient mixture) in the range of 80% - 120% of the test concentration specified in the method of analysis. The percentage recovery in presence of placebo and relative standard deviations for all the values of % recovery was reported.

1.4.2 Preparation of analytical solutions for recovery of Curcumin in presence of placebo:

a) **Blank** **:** mobile phase was used as blank.

b) **Standard preparation** **:** Prepare as described under **2.3**.

Preparation of recovery solutions in presence working standard of placebo in diazen Tablets

1) Preparation of 80% recovery solution: Transferred an accurately weighed quantity of about 4mg of Curcumin working standard into a 200 ml volumetric flask containing 8.098 g of placebo, 40 ml of tetra hydro furan was added and sonicated for 20 minutes and the volume was made up with mobile phase. Filtered through 0.45 µm membrane filter. **(Prepared in triplicate).**

2) Preparation of 100% recovery solution: Transferred an accurately weighed quantity of about 5 mg of Curcumin working standard into 200 ml volumetric flask containing 8.098 g of placebo, 40 ml of tetra hydro furan was added and sonicated for 20 minutes and the volume was made up with mobile phase. Filtered through 0.45 µm membrane filter. **(Prepared in triplicate).**

3) Preparation of 120% recovery solution: Transferred an accurately weighed quantity of about 6mg of Curcumin working standard into a 200 ml volumetric flask containing 8.098 g of placebo, 40 ml of tetra hydro furan was added and Sonicated for 20 minutes and the volume was made up with mobile phase. Filtered through 0.45 µm membrane filter. **(Prepared in triplicate).**

Table-6: Sequence for recovery in presence of placebo of diazen tablet.

S. No.	Sample name	Number of injections
1	Blank	1
2	Standard preparation	6
3	Blank for carry over	1
4	80% recovery solution_1	3
5	80% recovery solution_2	3
6	80% recovery solution_3	3
7	Standard preparation (Bracketing)	1
8	100% recovery solution_1	3
9	100% recovery solution_2	3
10	100% recovery solution_3	3
11	120% recovery solution_1	3
12	120% recovery solution_2	3
13	120% recovery solution_3	3
14	Standard preparation (closing)	1

1.4.3 Procedure: Separately injected 20 µl of standard preparation in replicate and recovery solutions into the chromatograph and the peak responses for the major peaks was measured. The percentage recovery in recovery solutions was calculated using the following expression. (Refer **Table-6** for sequence of injections)

1.4.4 Calculations

$$\frac{AT}{AS} \times \frac{WS}{200} \times \frac{5}{50} \times \frac{200}{1} \times \frac{100}{10}$$

= _____mg of Curcumin WS recovered

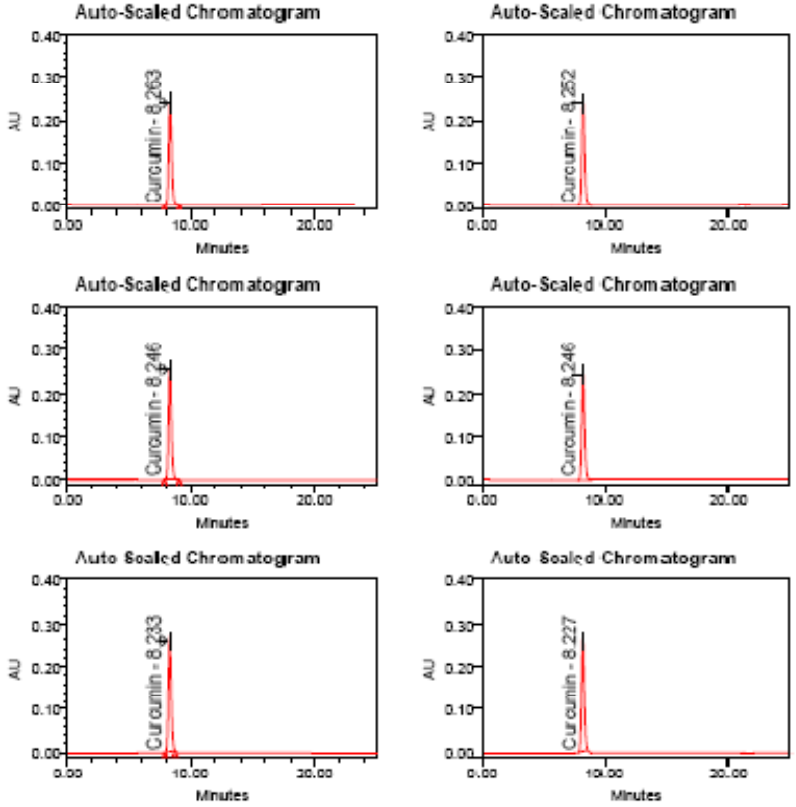
$$\% \text{ of recovery} = \frac{\text{mg of Curcumin WS recovered}}{\text{mg of Curcumin WS added}} \times 100$$

1.4.5 Acceptance criteria: The percentage recovery should lie between 98% and 102%. The relative standard deviation of all recovery values should not be more than 2.0%.

Chromagram-25 Standard preparation for recovery

SAMPLE INFORMATION

Sample Name:	CURCUMIN STANDARD	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 1:06:06 PM IST, 12/19/2011 1:31:52 PM IST, 12/19/2011 1:57:40 PM IST, 12/19/2011		
Date Processed:	1/6/2012 10:36:38 AM IST, 1/6/2012 10:36:40 AM IST, 1/6/2012 10:36:46 AM IST, 1/6/2012		



Component Summary Table
Name: Curcumin

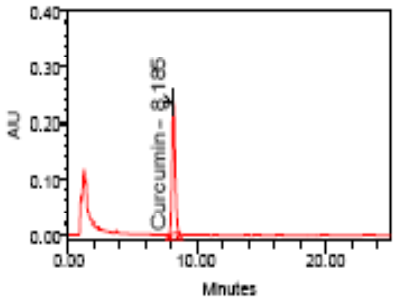
	Sample Name	Name	RT	Area
1	CURCUMIN STANDARD	Curcumin	8.263	4185602
2	CURCUMIN STANDARD	Curcumin	8.252	4154623
3	CURCUMIN STANDARD	Curcumin	8.246	4324699
4	CURCUMIN STANDARD	Curcumin	8.246	4200032
5	CURCUMIN STANDARD	Curcumin	8.233	4279649
6	CURCUMIN STANDARD	Curcumin	8.227	4257262
Mean			8.245	4233644.426
Std. Dev.			0.013	64260.762
% RSD			0.2	1.5

Chromagram-26 80% of assay preparation-1 for recovery

SAMPLE INFORMATION

Sample Name:	80% RECOVERY SOLUTION-1	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	3	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 4:08:43 PM IST, 12/19/2011 4:32:41 PM IST, 12/19/2011 4:58:40 PM IST		
Date Processed:	1/6/2012 10:49:21 AM IST, 1/6/2012 10:49:47 AM IST, 1/6/2012 10:52:20 AM IST		

Auto-Scaled Chromatogram



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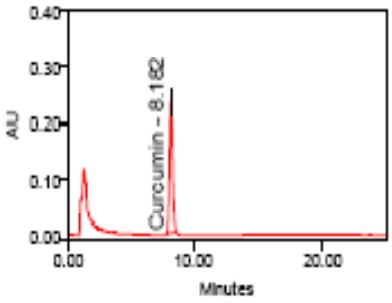

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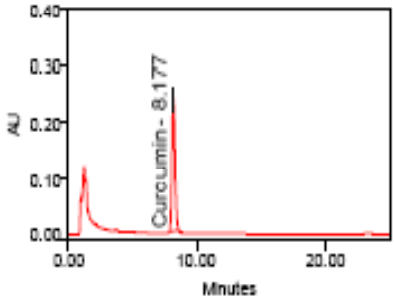


Component Summary Table
Name: Curcumin

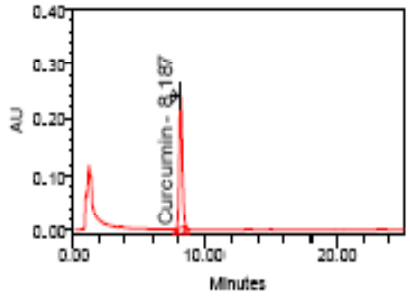
	Sample Name	Name	RT	Area
1	80% RECOVERY SOLUTION-1	Curcumin	8.185	3956176
2	80% RECOVERY SOLUTION-1	Curcumin	8.182	3977711
3	80% RECOVERY SOLUTION-1	Curcumin	8.177	3998348
Mean			8.181	3977411.901
Std. Dev.			0.004	21087.542
% RSD			0.0	0.5

Chromagram-27 80% of assay preparation-2 for recovery

SAMPLE INFORMATION

Sample Name:	80% RECOVERY SOLUTION-2	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	4	Processing Method:	Curcumin_PM
Injection#:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 5:24:22 PM IST, 12/19/2011 5:50:08 PM IST, 12/19/2011 6:15:54 PM IST		
Date Processed:	1/6/2012 11:08:16 AM IST, 1/6/2012 11:08:45 AM IST, 1/6/2012 11:09:15 AM IST		

Auto-Scaled Chromatogram



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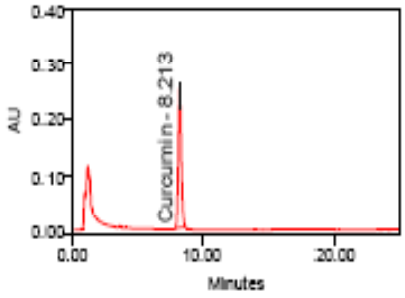

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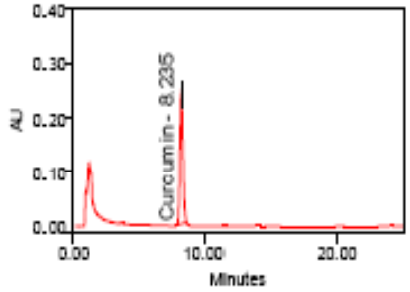


Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	80% RECOVERY SOLUTION-2	Curcumin	8.187	4040941
2	80% RECOVERY SOLUTION-2	Curcumin	8.213	4058112
3	80% RECOVERY SOLUTION-2	Curcumin	8.235	4071872
Mean			8.211	4056974.725
Std. Dev.			0.024	15496.668
% RSD			0.3	0.4

Chromagram-28 80% of assay preparation-3 for recovery

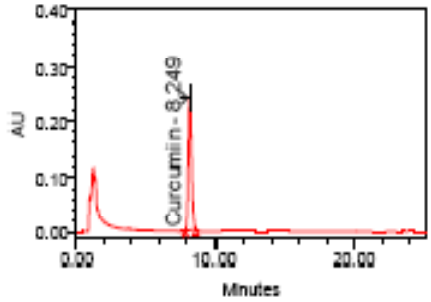
SAMPLE INFORMATION

Sample Name:	80% RECOVERY SOLUTION-3	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	5	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		

Date Acquired: 12/19/2011 6:41:37 PM IST, 12/19/2011 7:07:23 PM IST, 12/19/2011 7:33:08 PM IST

Date Processed: 1/6/2012 11:13:19 AM IST, 1/6/2012 11:13:41 AM IST, 1/6/2012 11:14:04 AM IST

Auto-Scaled Chromatogram



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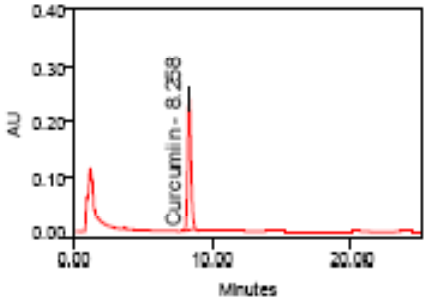

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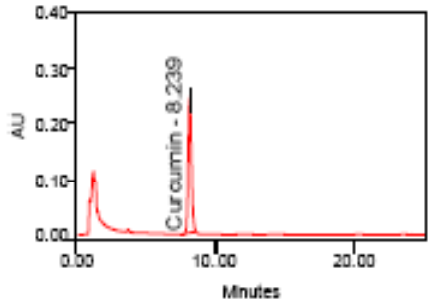


Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	80% RECOVERY SOLUTION-3	Curcumin	8.249	4081251
2	80% RECOVERY SOLUTION-3	Curcumin	8.258	4089738
3	80% RECOVERY SOLUTION-3	Curcumin	8.239	4097707
Mean			8.249	4089565.473
Std. Dev.			0.009	8229.289
% RSD			0.1	0.2

Calculation for 80% recovery preparation

ACCURACY

80% RECOVERY:

PREPARATION-1:

$$\frac{3977411.9}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

4.603 mg of curcumin WS recovered

% of recovery = 101.17 %

PREPARATION-2:

$$\frac{4056974.73}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

4.696 mg of curcumin WS recovered

% of recovery = 101.63 %

PREPARATION-3:

$$\frac{4089565.48}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

4.733 mg of curcumin WS recovered

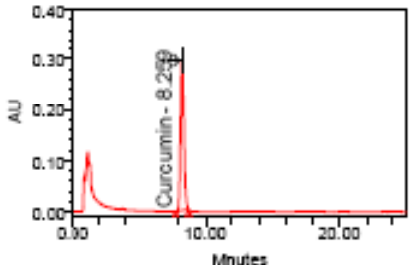
% of recovery = 101.79 %

Chromagram-29 100% of assay preparation-1 for recovery

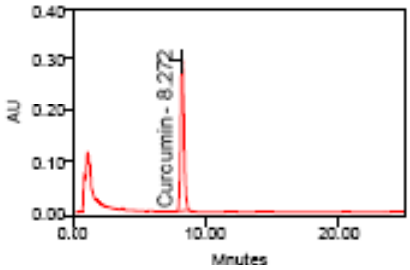
SAMPLE INFORMATION

Sample Name:	100% RECOVERY SOLUTION-1	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	6	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 8:24:45 PM IST, 12/19/2011 8:50:30 PM IST, 12/19/2011 9:16:19 PM IST		
Date Processed:	1/6/2012 11:15:03 AM IST, 1/6/2012 11:15:14 AM IST, 1/6/2012 11:15:37 AM IST		

Auto-Scaled Chromatogram



Auto-Scaled Chromatogram



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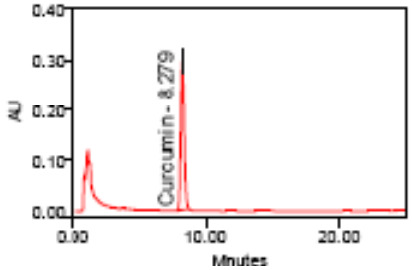


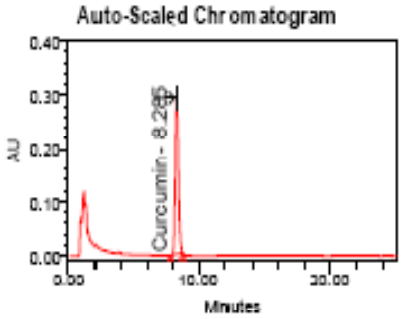
Component Summary Table
Name: Curcumin

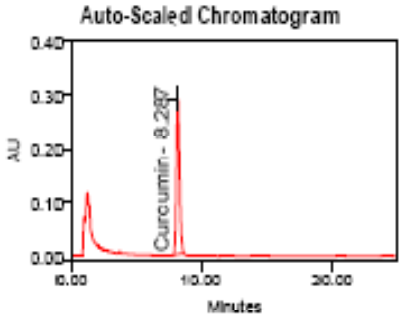
	Sample Name	Name	RT	Area
1	100% RECOVERY SOLUTION-1	Curcumin	8.259	5046160
2	100% RECOVERY SOLUTION-1	Curcumin	8.272	5089044
3	100% RECOVERY SOLUTION-1	Curcumin	8.279	5082003
Mean			8.270	5072402.324
Std. Dev.			0.010	22997.854
%RSD			0.1	0.5

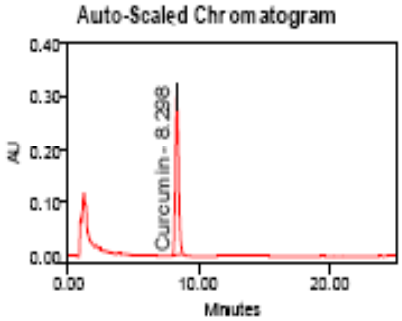
Chromagram-30 100% of assay preparation-2 for recovery

SAMPLE INFORMATION

Sample Name:	100% RECOVERY SOLUTION-2	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	7	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.000 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 9:42:02 PM IST, 12/19/2011 10:07:49 PM IST, 12/19/2011 10:33:36 PM IST		
Date Processed:	1/6/2012 11:19:43 AM IST, 1/6/2012 11:19:56 AM IST, 1/6/2012 11:20:12 AM IST		







Component Summary Table

Name: Curcumin

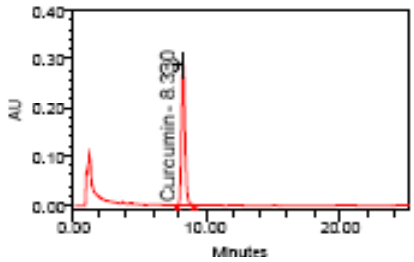
	Sample Name	Name	RT	Area
1	100% RECOVERY SOLUTION-2	Curcumin	8.295	5039693
2	100% RECOVERY SOLUTION-2	Curcumin	8.287	5052896
3	100% RECOVERY SOLUTION-2	Curcumin	8.298	5096615
Mean			8.293	5066401.189
Std. Dev.			0.007	28522.909
% RSD			0.1	0.6

Chromagram-31 100% of assay preparation-3 for recovery

SAMPLE INFORMATION

Sample Name:	100% RECOVERY SOLUTION-3	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	8	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/19/2011 10:59:22 PM IST, 12/19/2011 11:25:08 PM IST, 12/19/2011 11:50:54 PM IST		
Date Processed:	1/6/2012 11:20:52 AM IST, 1/6/2012 11:21:01 AM IST, 1/6/2012 11:21:09 AM IST		

Auto-Scaled Chromatogram



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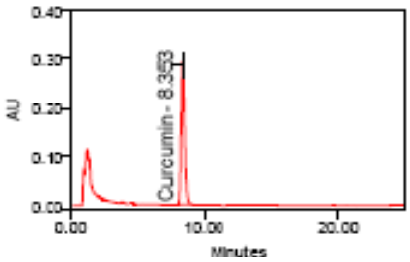

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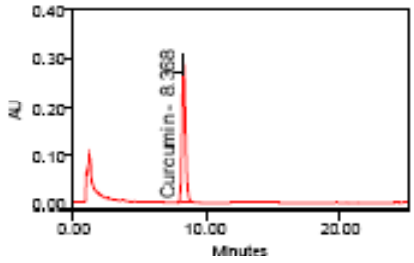


Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	100% RECOVERY SOLUTION-3	Curcumin	8.330	5098404
2	100% RECOVERY SOLUTION-3	Curcumin	8.353	5065773
3	100% RECOVERY SOLUTION-3	Curcumin	8.368	5049298
Mean			8.350	5071158.442
Std. Dev.			0.019	24991.982
% RSD			0.2	0.5

Calculation for 100% recovery preparation

ACCURACY

100% RECOVERY:

PREPARATION-1:

$$\frac{5072402.32}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

5.871 mg of curcumin WS recovered

% of recovery = 101.92 %

PREPARATION-2:

$$\frac{5066401.19}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

5.864 mg of curcumin WS recovered

% of recovery = 101.98 %

PREPARATION-3:

$$\frac{5071158.44}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

5.869 mg of curcumin WS recovered

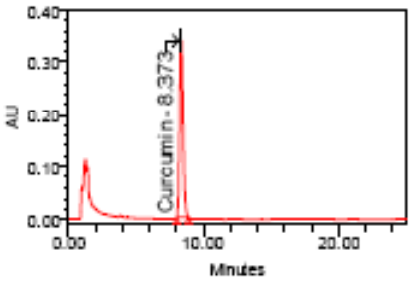
% of recovery = 101.90 %

Chromagram-32 120% of assay preparation-1 for recovery

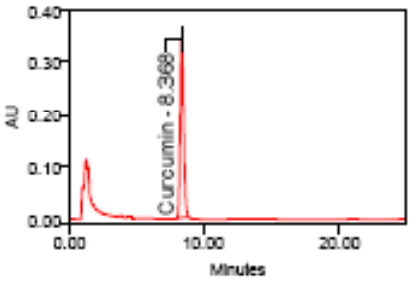
SAMPLE INFORMATION

Sample Name:	120% RECOVERY SOLUTION-1	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	9	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/20/2011 12:16:40 AM IST, 12/20/2011 12:42:27 AM IST, 12/20/2011 1:08:10 AM IST		
Date Processed:	1/6/2012 11:22:31 AM IST, 1/6/2012 11:22:49 AM IST, 1/6/2012 11:23:09 AM IST		

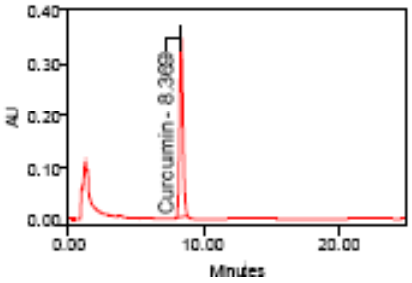
Auto-Scaled Chromatogram



Auto-Scaled Chromatogram



Auto-Scaled Chromatogram



Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area
1	120% RECOVERY SOLUTION-1	Curcumin	8.373	6008627
2	120% RECOVERY SOLUTION-1	Curcumin	8.368	6027008
3	120% RECOVERY SOLUTION-1	Curcumin	8.369	5994736
Mean			8.370	6010123.781
Std. Dev.			0.003	16187.887
% RSD			0.0	0.3

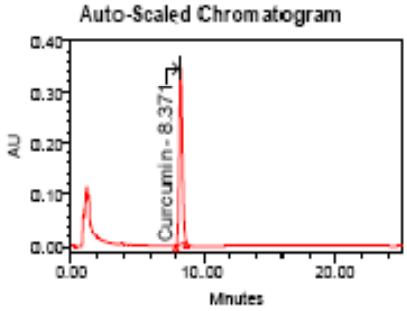
Chromagram-33 120% of assay preparation-2 for recovery

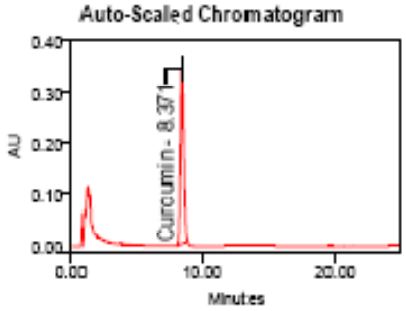
SAMPLE INFORMATION

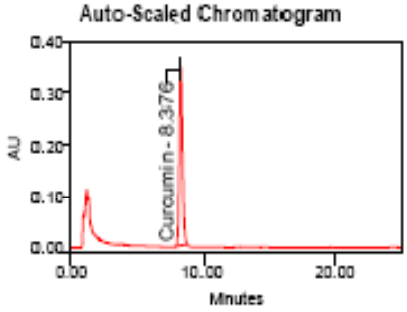
Sample Name:	120% RECOVERY SOLUTION-2	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	10	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		

Date Acquired: 12/20/2011 1:33:56 AM IST, 12/20/2011 1:59:43 AM IST, 12/20/2011 2:25:29 AM IST

Date Processed: 1/6/2012 11:28:58 AM IST, 1/6/2012 11:29:15 AM IST, 1/6/2012 11:29:31 AM IST







Component Summary Table

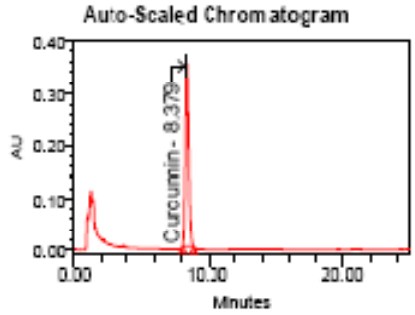
Name: Curcumin

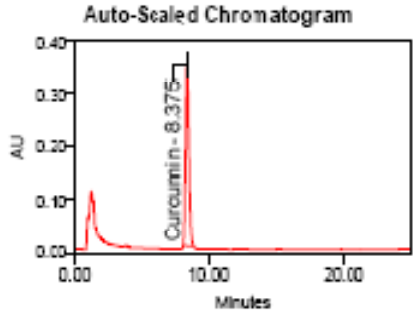
	Sample Name	Name	RT	Area
1	120% RECOVERY SOLUTION-2	Curcumin	8.371	5984287
2	120% RECOVERY SOLUTION-2	Curcumin	8.371	5982521
3	120% RECOVERY SOLUTION-2	Curcumin	8.376	6078444
Mean			8.373	6015083.986
Std. Dev.			0.003	54878.102
% RSD			0.0	0.9

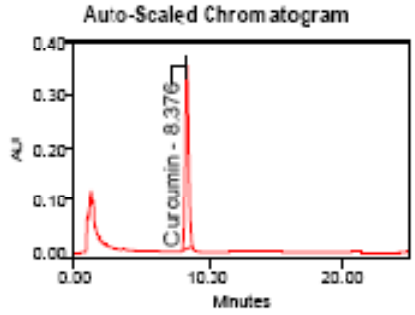
Chromagram-34 120% of assay preparation-3 for recovery

SAMPLE INFORMATION

Sample Name:	120% RECOVERY SOLUTION-3	Acquired By:	Yuvaraj
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	11	Processing Method:	Curcumin_PM
Injection #:	1 2, 3	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	26.0 Minutes		
Date Acquired:	12/20/2011 2:51:16 AM IST, 12/20/2011 3:17:02 AM IST, 12/20/2011 3:42:48 AM IST		
Date Processed:	1/6/2012 11:30:25 AM IST, 1/6/2012 11:30:37 AM IST, 1/6/2012 11:30:53 AM IST		







Component Summary Table

Name: Curcumin

	Sample Name	Name	RT	Area
1	120% RECOVERY SOLUTION-3	Curcumin	8.379	5993502
2	120% RECOVERY SOLUTION-3	Curcumin	8.375	5971470
3	120% RECOVERY SOLUTION-3	Curcumin	8.376	5981779
Mean			8.376	5982250.328
Std. Dev.			0.002	11023.682
% RSD			0.0	0.2

Calculation for 120% recovery preparation

ACCURACY

120% RECOVERY:

PREPARATION -1:

$$\frac{6010123.78}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

6.956 mg of curcumin WS recovered

% of recovery = 101.40 %

PREPARATION -2:

$$\frac{6015083.98}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

6.962 mg of curcumin WS recovered

% of recovery = 101.63 %

PREPARATION -3:

$$\frac{5982250.33}{4233644.43} \times \frac{24.5}{100} \times \frac{5}{50} \times \frac{200}{1}$$

6.924 mg of curcumin WS recovered

% of recovery = 101.52 %

1.5 SYSTEM SUITABILITY

1.5.1

Objective: The suitability of chromatographic system was determined by the method of analysis by establishing system suitability test parameters like relative retention times, resolution, tailing factor and number of theoretical plates for system suitability preparation and % RSD of Curcumin standard preparation. This parameters needs to be observed on daily basis.

1.5.2

Procedure: Separately injected 20 µl of standard preparation (6.1.3.) in to the chromatograph and the chromatogram was recorded and the relative retention times, tailing factor and number of theoretical plates and % RSD of standard preparation were measured.

1.5.3

Calculation: The relative retention times & tailing factor were calculated for system suitability preparation and % RSD for replicate injections of standard preparation

1.5.4

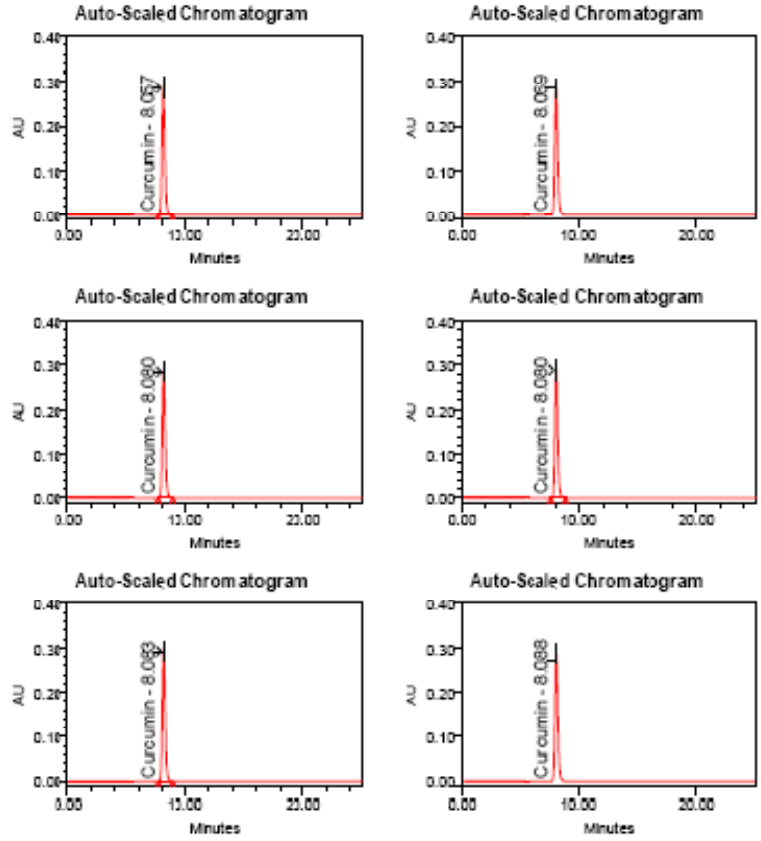
Acceptance criteria:

a) Peak symmetry as tailing factor for Curcumin peak for standard preparation should not be more than 2.0.
b) % RSD for standard preparation should not be more than 2.0%.Column efficiency as number of theoretical plates for Curcumin peak for standard preparation should not be less than 2000.

Chromagram-35

SAMPLE INFORMATION

Sample Name:	CURCUMIN STANDARD	Acquired By:	Ravi
Sample Type:	Standard	Acq. Method Set:	CURCUMIN_MSET
Vial:	2	Processing Method:	Curcumin_PM
Injection #:	1, 2, 3, 4, 5, 6	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 1:11:26 PM IST, 12/17/2011 1:37:11 PM IST, 12/17/2011 2:02:57 PM IST, 12/17/2011		
Date Processed:	1/7/2012 10:12:33 AM IST, 1/7/2012 10:13:34 AM IST, 1/7/2012 10:13:37 AM IST, 1/7/2012		



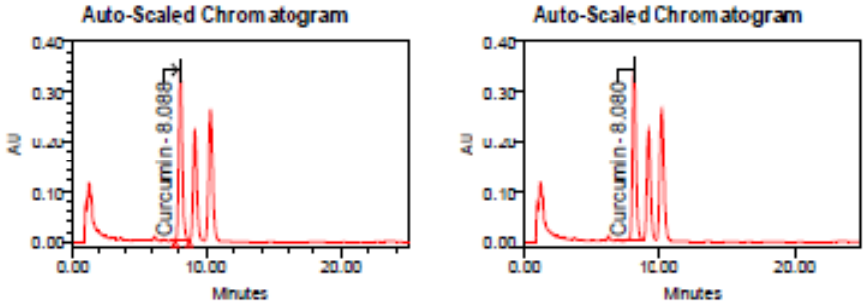
Component Summary Table
Name: Curcumin

	Sample Name	Name	RT	Area	USP Tailing	USP Plate Count
1	CURCUMIN STANDARD	Curcumin	8.057	4801791	1.197	5430.707
2	CURCUMIN STANDARD	Curcumin	8.069	4806736	1.201	5396.333
3	CURCUMIN STANDARD	Curcumin	8.080	4816578	1.203	5363.518
4	CURCUMIN STANDARD	Curcumin	8.080	4823163	1.195	5491.480
5	CURCUMIN STANDARD	Curcumin	8.083	4847330	1.190	5537.575
6	CURCUMIN STANDARD	Curcumin	8.088	4861349	1.207	5348.555
Mean			8.076	4826157.894	1.2	5428.0
Std. Dev.			0.011	23487.867	0.0	74.2
% RSD			0.1	0.5	0.5	1.4

Chromagram-36

SAMPLE INFORMATION

Sample Name:	ASSAY PREPARATION	Acquired By:	Ravi
Sample Type:	Unknown	Acq. Method Set:	CURCUMIN_MSET
Vial:	4	Processing Method:	Curcumin_PM
Injection #:	1, 2	Channel Name:	W2489 ChA
Injection Volume:	20.00 ul		
Run Time:	25.0 Minutes		
Date Acquired:	12/17/2011 5:03:23 PM IST, 12/17/2011 5:29:05 PM IST		
Date Processed:	1/7/2012 10:18:10 AM IST, 1/7/2012 10:18:39 AM IST		



	Sample name	Name	Rt	Area	USP tailing	USP plate count
1	Assay preparation-1	Curcumin	8.80	5858607	1.234	5218.183
2	Assay preparation-2	Curcumin	8.088	5870489	1.206	5229.859
Mean			8.084	5869563.068	1.2	5224.0
Std.dev			0.006	1267.568	00	8.3
%RSD			0.1	0.0	0.1	0.2

6. RESULTS AND DISCUSSION

The RP-HPLC method was developed for estimation of Curcumin in poly herbal tablet dosage form and validation was performed in accordance with ICH guidelines for the following parameters such as system suitability linearity and range, precision, accuracy, ruggedness and specificity. The summary of results obtained in analytical method development and validation were shown in table 1.

Table 1: Data and Results

Parameters	Acceptance	Results
System suitability	1.% RSD NMT 2% 2. Tailing factor NMT 2 3.Theoretical plates NLT 2000	0.5% 1.2 Passes
Specificity	No interference with blank and placebo preparation	No interference
Precision (Repeatability)	% RSD NMT 2%	0.3
Accuracy	98-102%	101.73
Linearity	Correlation co-efficient NLT 0.999	0.9994
Ruggedness	% RSD NMT 2%	0.2

Validation summary report:

The observations and results obtained for each of the parameters like system suitability, linearity and range, precision (repeatability), intermediate precision, specificity and ruggedness lies well within the acceptance criteria. So the given method was simple, specific, linear, precise, accurate and rugged and extensively used for the estimation Curcumin in poly herbal tablet dosage form.

Discussion:

The Reverse Phase High Performance Liquid Chromatography method was developed. Pure drug chromatogram was run in different mobile phases containing acetonitrile, tetrahydrofuron and 1% citric acid also by using acetic acid. Different columns like C_8 & C_{18} were used. The mobile phase with THF: 1% citric acid in the ratio of 45:55 was used. Phenolex C_{18} analytical column was selected which gives a sharp peak and a symmetrical peak with 1.51 tailing. Calibration graph was found to be linear at range 20 µg/ml to 30 µg/ml. Five different concentrations of Curcumin in range above were prepared and 20 µl of each concentration injected in HPLC. The correlation of coefficient (r^2) was found to be 0.9994. It was observed that the concentration range showed a good relationship. The percentage assay or average amount of Curcumin in formulation was found to be 109.23% the low values of standard deviation and coefficient of variation at each level of the recovery experiment indicate high precision of the method.

CONCULSION

Curcumin is a (1*E*, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-Dione. It is an herbal drug which is used as an Anti microbial, Anti inflammatory, Hepato protective, Anti carcinogenic, Anti bacterial, Anti oxidant, Anti mutagenic. A simple, sensitive and cost effective RP-HPLC method with better system suitability parameters has been developed for the estimation of Curcumin in formulation. This method can be employed for the estimation of formulations containing poly herbal combinations.

From the experimental data results and parameters it was concluded that the developed RP-HPLC method has the following advantages:

- The standard and sample preparation requires less time
- No tedious extraction procedure was involved in the analytical process.
- Suitable for the analysis of raw materials, marketing samples and formulations.
- Hence, the chromatographic method developed for Curcumin was found to be simple, precise, accurate and cost effective and it could be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies.

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